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Departamento de Biología Celular, Fisiología e Inmunología

TESIS DOCTORAL

NOVEL REGULATORY PATHWAYS IN THE CENTRAL CONTROL OF PUBERTY: ANALYSIS OF THE ROLE OF CERAMIDE SIGNALING AND Mkrn3/miR-30b SYSTEM

Violeta Heras Domínguez

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NOVEL REGULATORY PATHWAYS IN THE CENTRAL CONTROL OF PUBERTY: ANALYSIS OF THE ROLE OF CERAMIDE SIGNALING AND Mkrn3/miR-30b SYSTEM

Memoria de Tesis Doctoral presentada por **Violeta Heras Domínguez**, licenciada en Biotecnología por la Universidad de Salamanca, para optar al grado de **Doctora** en Biomedicina por la Universidad de Córdoba.

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TÍTULO DE LA TESIS: NOVEL REGULATORY PATHWAYS IN THE CENTRAL CONTROL OF PUBERTY: ANALYSIS OF THE ROLE OF CERAMIDE SIGNALING AND Mkrn3/miR-30b SYSTEM

DOCTORANDO/A: Violeta Heras Domínguez

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo de Tesis Doctoral titulado "*Novel Regulatory Pathways in the Central Control of Puberty: Analysis of the Role of Ceramide Signaling and Mkrn3/miR-30b System*" ha sido completado de forma muy satisfactoria por la doctoranda Violeta Heras Domínguez en la Sección de Fisiología del Departamento de Biología Celular, Fisiología e Inmunología de la Universidad de Córdoba, entre los años 2012 y 2018, bajo nuestra dirección. El objetivo general de este trabajo ha sido la caracterización de nuevos mecanismos de regulación implicados en el control fisiológico de la pubertad, tales como la señalización central por ceramidas y la ruta hipotalámica miR-30b/ Mkrn3, así como su posible implicación en las alteraciones del desarrollo puberal comúnmente asociadas a condiciones desfavorables (especialmente aquellas de naturaleza metabólica).

La doctoranda no sólo ha cumplido ampliamente el objetivo general propuesto, sino que también ha aprovechado esta etapa formativa para (i) adquirir una considerable destreza en el manejo de diferentes técnicas de biología molecular y neuroendocrinología experimental, (ii) reforzar sus conocimientos en el área de estudio, (iii) estimular su pensamiento crítico y (iv) colaborar activamente en líneas de investigación estrechamente relacionadas con su línea de Tesis Doctoral. La excelente labor investigadora de la doctoranda durante este periodo se ha traducido hasta ahora en: (i) 13 comunicaciones a congresos nacionales e internacionales, entre las que destacan una

ponencia oral en el "20th European Congress of Endocrinology" (ECE 2018); (ii) 2 premios: Premio a la Mejor Comunicación Oral en la "XI Jornada de Jóvenes Investigadores del IMIBIC" por el trabajo titulado *"The Hypothalamic miR-30b/Mkrn3 Pathway is a Novel Central Regulator of Puberty Onset"* (2018) y el ESE Young Investigator Award en el "20th European Congress of Endocrinology" (ECE 2018) por el trabajo titulado *"Novel role of central ceramide signaling in mediating obesity-induced precocious puberty"*; (iii) 1 capítulo de libro como segunda autora titulado *"Female Puberty Overview"*, publicado en la segunda edición del libro "*Encyclopedia of Reproduction*" (Elsevier); (iv) 7 artículos científicos como coautora: 2 en primer decil (*Nature Communications* y *PNAS*), 3 en primer cuartil (2 en *Scientific Reports* y 1 en *Metabolism*) y 2 en segundo cuartil (*Endocrinology*, con índice de impacto de 5 años >4); y (v) dos artículos adicionales como primera autora, uno de los cuales se encuentra en revisión en una revista de primer decil, y el otro se anticipa será completado en su elaboración en breve a fin de proceder con su envío a evaluación, igualmente a una revista de primer decil, en el último trimestre del año en curso.

Por todo lo anteriormente expuesto, se autoriza la presentación de la tesis doctoral.

Córdoba, 25 de Octubre de 2018

Firma del/de los director/es

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A Mis Padres y Hermano
A Alejandro

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SUMMARY

SUMMARY

1. Introduction

Puberty is a crucial and complex developmental event characterized by the acquisition of reproductive capacity and sexual and somatic maturation[1]. Nowadays, it is well established that pubertal timing is under the control of sophisticated regulatory mechanisms highly sensitive to metabolic and environmental factors. However, our knowledge about these mechanisms and their potential involvement in pubertal alterations is still incomplete. The importance to unveil new molecular and neuroendocrine pathways for the central control of puberty is illustrated by recent epidemiological studies conducted in Europe and USA that have documented a trend for advanced puberty; a phenomenon related to higher risk of suffering other cardiovascular and metabolic diseases (e.g. hypertension, obesity, and diabetes).

On the above basis, it is important to notice that the activation of the reproductive axis at puberty depends on the degree of energy reserves of the organism[2]. In the last few years, the mechanisms involved in the integrative control of the energy balance and pubertal development have been extensively studied. These mechanisms seemingly involve a large array of metabolic hormones and neuropeptides, which impinge and integrate at the hypothalamic centers controlling the reproductive axis[3,4]. Among those regulatory elements, the metabolic hormones leptin and ghrelin, as stimulatory and inhibitory signals of puberty onset, respectively, and the puberty-activating neuropeptide, kisspeptin, as a central conduit for transmitting the pubertal actions of different metabolic hormones to the reproductive brain, have proven to be especially relevant[3,5-7]. Yet, our knowledge about the mechanism(s) of action of these (and others) regulatory factors in the central control of puberty and their potential contribution to alterations in the timing of puberty is still incomplete.

Interestingly, **ceramides**, a family of sphingolipids of ubiquitous nature involved in different cellular processes, have been recently proposed as hypothalamic mediators in the control of energy homeostasis and metabolic disorders[8-10]. In particular, high hypothalamic levels of ceramides have been reported to block the anorexigenic effects of leptin[11], while they might mediate the orexigenic roles of ghrelin in the control of food intake and energy balance[12]. However, the potential role of hypothalamic ceramides in the central control of puberty onset, as well as their putative interactions with other relevant neuroendocrine factors, such as kisspeptins or leptin, in this context, remain fully unexplored.

On the other hand, it is worth to note that novel targets and regulatory mechanisms have been recently identified in the context of the central control of puberty. Among them, the maternally imprinted gene encoding the makorin RING-finger protein 3, **Mkrn3**, as a novel target, and miRNAs,

as a novel regulatory mechanism, seems to have a relevant role[13-15]. Recent evidence has suggested that Mkrn3 may act as a potential repressor of puberty onset. This is based on (i) the association of deleterious mutations of MKRN3 with central precocious puberty in boys and girls[16-22]; (ii) the decrease in circulating levels of Mkrn3 detected in both sexes before puberty onset[23-25]; and (iii) the significant reduction of the hypothalamic Mkrn3 expression observed during the juvenile-pubertal transition in rodents[16,26]. Despite such evidence, the regulatory mechanisms whereby Mkrn3 is precisely controlled during postnatal/pubertal maturation and their biological actions in normal and altered puberty are totally unknown. Interestingly, bioinformatic analyses conducted in our group have revealed that the microRNA miR-30b shows three predicted and conserved binding sites at the 3′ untranslated region (3′-UTR) of Mkrn3. Whether such miRNA contributes to the regulation of Mkrn3 expression in the central control of puberty has not been explored so far.

Based on the above, the main objective of this Doctoral Thesis is to characterize novel regulatory mechanisms involved in the physiological control of puberty, such as central ceramide signaling and the miR-30b/Mkrn3 pathway, and to elucidate their potential involvement in the alterations of pubertal development frequently linked to unfavourable (mainly metabolic) conditions.

2. Research contents

In the experimental set 1#, a series of studies were implemented to analyze the putative role of central ceramide signaling in the metabolic control of puberty onset, using prepubertal female rats in normal conditions or subjected to obesogenic insults promoting precocious puberty (i.e. early overfeeding).

Our initial studies documented a significant increase in the hypothalamic levels of ceramides in early overfed female rats (ON) with precocious puberty. Based on these data, we decided to evaluate the involvement of perturbed central ceramide signaling in the etiopathogenesis of precocious puberty linked to early overfeeding. For this purpose, prepubertal female rats were intracerebroventricularly injected with a cell-penetrating precursor of the *de novo* ceramides synthesis, called CER C6, resulting in advanced puberty onset. In contrast, the chronic inhibition of central ceramide synthesis with myriocin (MYR) caused a significant delay of puberty. Importantly, none of the two treatments altered body weight, food intake or gonadotropin levels.

Next, we analyzed the potential interaction between central ceramide signaling and kisspeptin or leptin in the timing of puberty in a model of delayed puberty induced by chronic undernutrition (25%), in which endogenous kisspeptin and leptin levels are suppressed, and hence the effects of "rescue" experiments by administration of the exogenous factors can be readily detected. Treatment with either kisspeptin or leptin partially rescued delayed puberty. Conversely, such stimulatory effects, especially those derived from kisspeptin, were largely prevented by co-administration with the ceramide inhibitor, MYR, thus suggesting that central ceramide signaling mediates part of the

stimulatory effects of kisspeptin and, to a lesser degree, leptin on puberty onset. However, the fact that MYR did not alter *Kiss1* mRNA expression in two relevant hypothalamic populations for the reproductive actions of kisspeptin, such as the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC), or did it attenuate kisspeptin-induced GnRH/LH responses ex vivo or in vivo, respectively, suggested that the pubertal actions of central ceramides might require the involvement of an alternative non-neuroendocrine pathway.

In this context, additional studies were focused in deciphering the potential role of central ceramide signaling in the modulation of an alternative Paraventricular (PVN)-ovarian sympathetic pathway that might lead to the onset of puberty and, eventually, contributes to obesity-induced precocious puberty. Our results suggest that early-onset obesity advanced the maturation of the ovarian sympathetic tone in immature female rats (PND25), as evidenced by increased levels of relevant markers of sympathetic activity, such as noradrenaline (NE) and 3-Methoxy-4-Hydroxyphenylglycol (MHPG) in the celiac ganglia and the ovary, as well as Ngf/Ngfr ovarian signaling. Interestingly, the blockade of central ceramide synthesis with MYR in early overfed female rats resulted in a partial normalization of the timing of puberty, in terms of vaginal opening and first estrus, which are considered external markers of puberty onset and ovulation, respectively. Furthermore, the ovarian sympathetic activity was partially normalized in those animals, which showed lower levels of NE in the celiac ganglia and the ovary, as well as decreased ovarian Ngf/Ngfr signaling.

Remarkably, we obtained neuroanatomical evidence that supports the potential role of ceramides synthesis at the PVN, considered as the hypothalamic start-point of the ovarian sympathetic pathway, in the precocious puberty linked to early-onset obesity. In particular, the expression of serine palmitoyltransferase (SPTLC-1), the gene encoding the first enzyme of the *de novo* synthesis of ceramides, was significantly increased in the PVN of early overfed rats with advanced puberty. Additionally, our data show that the number of kisspeptin-ir fibers is significantly reduced in the PVN of those animals, thus suggesting its potential relevance in such context.

In the experimental set 2#, different studies were performed in rodent models to evaluate the putative physiological role of miR-30b/Mkrn3 system in the central control of puberty. Hypothalamic expression profiles of Mkrn3 and miR-30b were analyzed during normal postnatal maturation, and in preclinical models of altered puberty, such as early (neonatal and/or infantile) manipulation of feeding (20 pups/litter), sex steroid milieu, and photoperiod (constant darkness during 5-10 days). At the hypothalamic level, our data document a decrease in Mkrn3 expression along postnatal maturation. Conversely, miR-30b levels displayed an opposite expression pattern, with minimal neonatal levels and progressive increases along postnatal development. In addition, our results show that neonatal estrogenization and early postnatal underfeeding, two models of perturbed puberty, alter the hypothalamic ratios of miR-30b/Mkrn3 at the expected time of puberty and the early

infantile period, respectively, in female rats. Furthermore, in vitro assays, based on heterologous expression of a reporter vector harboring the 3′-UTR of mouse Mkrn3 in HEK-293 cells, showed that miR-30b represses the transcriptional activity of *Mkrn3*. In the same vein, timely (juvenile) blockade of miR-30b binding to its seeds regions at the 3′-UTR of Mkrn3 in vivo, by central infusion of tailored target site blockers, reversed the prepubertal down-regulation of hypothalamic Mkrn3 protein and delayed female puberty.

3. Conclusions

The main conclusions of our studies are the following:

1. Ceramide signaling constitutes a novel pathway for the central control of pubertal timing, which mediates at least part of the regulatory actions of kisspeptins (and to a lesser extent leptin), likely via a GnRH-independent pathway, involving the PVN and ovarian sympathetic innervation.

2. This kisspeptin-ceramide pathway at the PVN plays a relevant pathophysiological role in the generation of pubertal precocity associated to early-onset obesity.

3. The miRNA, miR-30b, is a novel central regulator of the puberty-repressing factor, Mkrn3, acting at highly conserved regions at the 3′-UTR of this gene.

4. This miR-30b/Mkrn3 pathway seemingly plays a distinct role in the physiological control of the timing of puberty, and its perturbations in conditions of early nutritional or hormonal alterations.

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RESÚMEN

1. Introducción o motivación de la tesis

La pubertad es un evento clave y complejo que acontece durante el desarrollo caracterizado por una maduración somática y sexual que culmina con la adquisición de la capacidad reproductiva (1). Hoy en día se sabe que el tiempo de llegada de la pubertad está sometido a un mecanismo de regulación muy preciso y sofisticado altamente sensible a factores metabólicos y medioambientales. Sin embargo, nuestro conocimiento sobre estos mecanismos y su papel esencial en alteración puberal son en gran medida desconocidos.

La importancia de desvelar nuevas rutas moleculares y neuroendocrinas para el control central de la pubertad radica en estudios epidemiológicos recientes llevados a cabo en Europa y EEUU que documentan una tendencia incrementada a la pubertad adelantada, un fenómeno relacionado con alto riesgo de sufrir enfermedades cardiovasculares y metabólicas (hipertensión, obesidad y diabetes).

En este sentido cabe destacar que la activación del eje reproductivo en pubertad depende del grado de reservas energética del organismo (2). En los últimos años, se ha profundizado en el estudio de los mecanismos involucrados en el control integral del balance energético y el desarrollo puberal. Estos mecanismos parecen implicar a una gran lista de hormonas metabólicas y neuropéptidos, que se integran a nivel de los centros hipotalámicos controlando el eje reproductivo. Entre ellos, destaca el papel de las hormonas metabólicas Leptina y Ghrelina, como señales estimuladoras e inhibidoras respectivamente, así como el neuropéptido activador de la pubertad, Kisspeptina, como elemento central en la transmisión de acciones puberales de diferentes hormonas metabólicas en el cerebro reproductivo (3,5-7). Sin embargo, nuestros conocimientos sobre los mecanismos de acción de estos y otros factores reguladores en el control central de la pubertad y su contribución en alteraciones puberales permanece aún por descubrir.

Estudios recientes han propuesto a la señalización central de Ceramidas, una familia de esfingolípidos de naturaleza ubicua, como nuevos mediadores hipotalámicos en el control de la homeostasis energética y alteraciones metabólicas (8-10).

En particular, elevados niveles hipotalámicos de Ceramidas inhiben los efectos anorexigénicos de la Leptina (11) mientras que estimulan los efectos orexigénicos de Ghrelina en el control de la ingesta y el balance energético (12). Sin embargo la posible participación de la señalización central de ceramidas en la regulación metabólica de la pubertad así como una posible interacción con otros elementos neuroendocrinos relevantes en la activación del eje reproductor tales como Kisspeptina, no han sido explorados hasta la fecha.

Por otro lado, merece mención especial la identificación de nuevas dianas y mecanismos reguladores identificados en el contexto del control central de la pubertad. En este sentido, el gen maternalmente imprintado, makorin RING-finger protein 3, Mkrn3, y miRNAs como nuevos mecanismos

reguladores, parecen tener un papel destacado (13-15). MKRN3 actúa como un potencial represor transcripcional en el control central de la pubertad debido a i) La asociación de mutaciones inactivantes de dicho gen con pubertad precoz central en niños y niñas (16-22), (ii) Los niveles circulantes de MKRN3 disminuyen en suero de pacientes tanto en niños como en niñas antes de la llegada a pubertad (23-25) y iii) Niveles hipotalámicos de Mkrn3 en ratón macho y hembra se encuentran significativamente reducidos durante la transición juvenil-puberal (16,26). Pese a estas evidencias, los mecanismos reguladores por los que Mkrn3 es controlada de forma precisa durante la maduración postnatal/puberal y sus acciones biológicas en pubertad normal y alterada son totalmente desconocidos. Análisis bioinformáticos conducidos en nuestro grupo han revelado que la familia de microRNAs miR-30 muestran tres sitios de unión altamente conservados en la región no traducida 3' (3'UTR) de la Mkrn3. Si tales microRNAs contribuyen a la regulación de la expresión de Mkrn3 en el control central de la pubertad no ha sido estudiado hasta la fecha.

Teniendo en cuenta los antecedentes previos expuestos, el objetivo general de esta Tesis Doctoral fue caracterizar nuevos mecanismos reguladores implicados en el control fisiológico de la pubertad, tales como el papel de la señalización central de ceramidas así como la caracterización del sistema miR-30b/Mkrn3 en las alteraciones del desarrollo puberal frecuentemente unidas a condiciones (principalmente metabólicas) desfavorables.

2.Contenido de la investigación

En el primer bloque de experimentos encaminados a analizar el posible papel de la señalización central de ceramidas en el control metabólico de la pubertad se empleó un modelo de ratas hembra con pubertad adelantada asociada a obesidad temprana. Nuestros estudios iniciales documentaron un incremento significativo en los niveles hipotalámicos de ceramidas en este modelo. En base a estos datos, decidimos evaluar el papel de la señalización central de ceramidas en la etiopatogénesis de pubertad precoz asociada a obesidad temprana. Para ello, ratas hembra prepuberales fueron inyectadas intracerebroventricularmente con un precursor de la síntesis the de novo de ceramidas, CER C6, lo que resultó en una pubertad adelantada. Por el contrario, la inhibición crónica de la síntesis central de ceramidas usando myriocin (MYR) produjo un retraso significativo de pubertad. Ninguno de los dos tratamientos alteró el peso corporal, la ingesta y los niveles de gonadotropinas.

Seguidamente, analizamos la posible interacción entre la señalización de ceramidas y kispeptina o leptina en el tiempo de llegada a pubertad en un modelo de pubertad retrasada inducido por una subnutrición crónica (25%) , dónde los niveles endógenos de kispeptina o leptina fueron suprimidos y por tanto los efectos de "rescate" por administración de factores exógenos puede ser fácilmente detectado.

El tratamiento con kispeptina o leptina parcialmente rescató la pubertad. Por el contrario, tal efecto estimulador, especialmente los derivados del tratamiento con kisspeptina, fueron altamente

prevenidos por coadministración con el inhibidor de ceramidas, MYR, por tanto sugiriendo que la señalización central de ceramidas media parte de los efectos estimuladores de kispeptina y en menor grado de leptina en el tiempo de llegada a pubertad.

Sin embargo, el hecho de que MYR no alteró la expresión de Kiss1 en las dos poblaciones hipotalámicas relevantes para las acciones reproductivas de kisspeptina tales como el núcleo anteroventral periventricular (AVPV) y el núcleo arcuato (ARC), ni tampoco atenuó las respuestas GnRH/LH inducidas por kispeptinas ex vivo o in vivo, sugirió que las acciones puberales de la señalización central de ceramidas requieren de la participación de sistemas alternativos no neuroendocrinos.

En este contexto, se realizaron estudios adicionales centrados en descifrar el papel de la señalización central de ceramidas en la modulación de una ruta alternativa Paraventricular-Inervación simpática del ovario que podría conducir el tiempo de pubertad y eventualmente, contribuir a la pubertad precoz asociada a obesidad temprana.

Nuestros resultados sugieren que la obesidad de inicio temprano adelanta la maduración del tono simpático ovárico en ratas hembra inmaduras de 25 días, como evidenció el incremento significativo de los niveles de los marcadores relevantes de la actividad simpática tales como noradrenalina (NE) y 3-Metoxi-4-Hidroxifenilglicol (MHPG) en el Ganglio celíaco (CG) y Ovario (OVA) así como la señalización ovárica Ngf/Ngfr.

Sorprendentemente, el bloqueo de la señalización central de ceramidas con MYR en ratas hembra sobrenutridas resultó en una normalización parcial del tiempo de pubertad, en términos de apertura vaginal y primer estro , que son considerados marcadores externos de pubertad y ovulación respectivamente. Adicionalmente, la actividad simpática ovárica también se normalizó de forma parcial en estos animales, mostrando niveles más bajos de NE en el ganglio celíaco y ovario, así como una disminución en la señalización de Ngf/Ngfr.

Remarcablemente, nuestros resultados demuestran evidencias neuroanatómicas que apoyan el papel de la síntesis de ceramidas en el PVN, considerado como punto de partida hipotalámico de la ruta simpática al ovario, en la pubertad precoz asociada a obesidad. En particular, la expression de la Serin palmitoil transferasa (SPTLC1), el gen que codifica la primera enzima de la síntesis the de novo de ceramidas, fue incrementada significativamente en el PVN de ratas obesas con pubertad precoz. Nuestros datos muestran que el número de fibras de kispeptina fue reducido significativamente en el PVN de estos animales obesos, por tanto sugiriendo su potencial importancia en este contexto.

En el segundo bloque de experimentos, se llevaron a cabo una serie de estudios en modelos de roedores para evaluar el papel fisiológico del sistema hipotalámico miR-30b/Mkrn3 en el control central de la pubertad.

Se analizaron perfiles de expresión hipotalámicos de Mkrn3 y miR-30b mRNA durante el desarrollo puberal normal y en modelos preclínicos de pubertad alterada, tales como manipulación (neonatal y/o infantil) temprana de subnutrición (20 crías/madre), esteroides sexuales, y fotoperíodo (oscuridad constante 5-10 días). A nivel hipotalámico, nuestros análisis de expresión documentan una disminución de los niveles de Mkrn3 a lo largo del desarrollo postnatal. Por el contrario, los niveles de miR-30b mostraron patrones de expresión opuesta, con mínimos niveles neonatales e incremento progresivo a lo largo del desarrollo. Por otro lado, los datos de estrogenización neonatal y una subnutrición temprana, dos modelos de pubertad alterada, modificaron los ratios hipotalámicos miR-30b/Mkrn3 en el tiempo esperado de pubertad y en el período infantil temprano, respectivamente en rata hembra.

Nuestros ensayos in vitro, basados en expresión heteróloga de un vector reportero que contiene la región 3' UTR de Mkrn3 de ratón en células HEK-293, mostró que miR-30b reprime la actividad transcripcional de Mkrn3. En el mismo sentido, El bloqueo en etapa juvenil de la unión de miR-30b a sus 3 regiones semilla en la región 3'UTR de Mkrn3 in vivo mediante la inyección central de una mezcla de "Target site Blockers, revirtió la desregulación prepuberal de la proteína hipotalámica Mkrn3 y retrasó la pubertad en la hembra.

3.Conclusiones

Las principales conclusiones derivadas de este studio son las siguientes:

1. La señalización central de Ceramidas constituye una nueva vía para el control central del tiempo de llegada a pubertad, mediando al menos parte de las acciones reguladoras de las Kispeptinas (y en menor medida Leptina) mediante señalización GnRH-independiente, involucrando al PVN y la inervación simpática ovárica.

2. El Sistema Kispeptina-Ceramida en el PVN juega un papel patofisiológico relevante en la generación de pubertad precoz asociada a obesidad temprana.

3. El microRNA, miR-30b, es un nuevo regulador central del factor supresor de la pubertad, Mkrn3, actuando en regiones altamente conservadas en el extremo 3' UTR de este gen.

4. Este Sistema miR-30b/Mkrn3 parece tener un papel destacado en el control fisiológico del tiempo de llegada a pubertad y en sus alteraciones en condiciones de estrés nutricional u hormonal temprano.

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ABBREBIATIONS

ABBREVIATIONS

- (X)-IR: X-immunoreactive
- 3′-UTR: 3′ untranslated region
- 3V: Third cerebral ventricle
- aa: Amino acid
- Acc: Accesory neurosecretoty nuclei
- AgRP: Agouti-related protein
- AMPK: AMP-activated protein kinase
- AMH: Anti-mullerian hormone
- ARC: Arcuate nucleus
- AVP: Arginine-vasopresine
- AVPV: Anterior ventral periventricular area
- BPS: Balano-preputial separation
- BW: Body weight
- cAMP: Cyclic adenosine monophosphate
- CART: Cocaine and Amphetamine-regulated transcript
- CD: Constant darkness
- CER C6: Ceramide precursor
- CERS: Ceramide synthases
- CERT: Ceramide transfer protein
- CG: Celiac Ganglia
- CNS: Central nervous system
- CPT1C: Carnitine palmitoyl transferase 1C
- CRH: Corticotropin-release hormone
- D1: Diestrus 1 or metestrus
- D2: Diestrus 2
- DAG: Diacylglycerol
- DB: Diagonal band of broca
- *db/db*: Leptin receptor-deficient mice
- DES: Dihydroceramide desaturases
- DHT: Dihydrotestosterone
- DMN: Dorsomedial nucleus
- Dyn: Dynorphin
- E: Estrus
- E_2: 17ß-estradiol
- EB: Estradiol benzoate
- EDCs: Endocrine-disrupting compounds
- FE: First Estrus
- FSH: Follicle-stimulating hormone
- GABA: γ-aminobutyric acid

- GAL: Galanin
- GALP: Galanin-like peptide
- GHS-R: GH secretagogue receptor
- Glu: Glutamate
- GnRH: Gonadotropin-releasing hormone
- GnRH-R: GnRH receptor
- GWAS: Genome wide asociation study
- hCG: Human chorionic gonadotropin
- HFD: Hight fat diet
- HH: Hypogonadotropic hypogonadism
- HPG: Hypothalamic-pituitary-gonadal
- icv: Intracerebroventricular
- IHC: Immunohistochemistry
- InfS: Infundibular (neural) Stalk
- ip: Intraperitoneal
- ISH: *in situ* hybridization
- KO: Knock-out
- KP-10: Kisspeptin-10
- KP-54: Kisspeptin-54
- LEP: Leptin
- LH: Luteinizing hormone
- LHA: Lateral hypothalamic area
- LH-R: LH/hCG receptor
- LL: Large litter
- MBH: Medio basal hypothalamus
- MHPG: 3-Methoxy-4 Hydroxyphenyl-glycol
- miRNA: MicroRNA
- Mkrn3: Makorin ring zinc finger protein3
- mRNA: Messenger RNA
- MSH: Melanocyte stimulating hormone
- mTOR: Mammalian target for rapamycin
- MYR: Myriocin
- NE: Noradrenaline
- Ngf: Neuronal growth factor
- Ngfr: Neuronal growth factor receptor
- NKB: Neurokinin B
- NN: Normonutrition
- NO: Nitric oxide
- NPY: Neuropeptide Y
- nt: Nucleotides
- *ob/ob*: Leptin-deficient mice

- Ob-R: Leptin receptor
- ON: Overnutrition
- ONP: Ovarian nerve plexus
- Ova: ovary
- OVX: Ovariectomized
- OW: Ovarian weight
- OX: Oxytocin
- P: Progesterone
- P75 NTR: Low-affinity neurotrophin receptor p75
- PaMC: Magnocellular neurons of the Paraventricular
- PaPC: Parvocellular neurons of Paraventricular
- PCR: Polymerase chain reaction
- PE: Proestrus
- PeP: Posterior paraventricular nucleus
- PeVN: Periventricular nucleus
- PND: Postnatal day
- POA: Preoptic area
- Pol II: Polymerase II
- POMC: Proopiomelanocortin
- PPit: Posterior lobe of the pituitary
- PR: Progesterone receptor
- pre-miRNA: Precursor miRNA
- PVN: Paraventricular nucleus
- RNA: Ribonucleic acid
- RP3V: Rostral periventricular area of the 3V
- sc: Subcutaneous
- Sch: Suprachiasmatic nucleus
- SEM: Standard error of the mean
- SL: Small litter
- SMases: Sphingomyelinases
- SMS: Sphingomyelin synthase
- SO: Supraoptic
- SON: Superior ovarian nerve
- SPTLC-1: Serine palmitoyltransferase long chain base subunit 1
- T: Testosterone
- TP: Testosterone propionate
- TrKA: Tyrosine kinase A
- TW: Testicular weight
- UW: Uterus weight
- VIP: Vasoactive intestinal peptide
- VO: Vaginal opening

- WT: Wild type
- ZNF127: Zinc finger protein 127
- α-MSH: α-melanocyte stimulating hormone
- ß-END: ß-endorphin

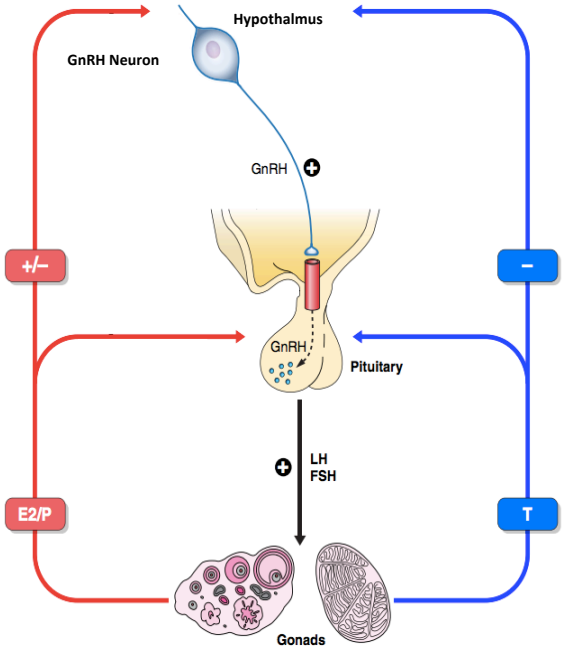
INTRODUCTION

INTRODUCTION

1. PUBERTY

Puberty is defined as a complex biological process involving sexual maturation, accelerated linear growth, psychological changes and the final acquisition of reproductive capacity[1]. Indeed, rather than a specific time-point in postnatal development, puberty is the culmination of a complex series of maturational events that start in utero and progress throughout early postnatal, infantile, and juvenile ages[27]. These events result in a diversity of phenotypic indices, whose timing is shaped by the dynamic interplay between our gene load and the environment. The success of this developmental process relies on the proper functional organization of the so-called hypothalamic-pituitary-gonadal (HPG) or gonadotropic axis, which is responsible for the completion of gonadal development and the attainment of sexual phenotypic maturity.

2. THE HYPOTHALAMIC-PITUITARY-GONADAL AXIS (HPG)



The function of the gonadotropic axis requires the dynamic interaction of three major groups of signals arising from (i) the hypothalamus, where a subset of neurons synthesize and release the decapeptide gonadotropin-releasing hormone (GnRH), (ii) the adenohypophysis, where gonadotropes secrete the gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone, and (iii) the gonads, which, in addition to producing fertilizable gametes from puberty onwards, are responsible for the release of sex steroids (i.e. estrogen, progestagens and testosterone) and peptides [28]. These major components of the HPG axis are connected via feed-forward and feedback loops, thus facilitating its homeostatic regulation. In this system, GnRH neurons hold a major hierarchical role and operate as the final output pathway for different regulatory signals, including central neuropeptides and neurotransmitters, as well as peripheral hormones[29] (**Figure 1**).

Figure 1: Schematic representation of the HPG axis. (Modified from *"Kisspeptins and Reproduction: Physiological Roles and Regulatory Mechanisms", L. Pinilla et al. Physiological Reviews, 2012*).

HYPOTHALAMUS

The hypothalamus acts as the central regulator of endocrine glands through neurotransmitters and neuropeptides secreted by different neuronal populations, which are involved in physiological activities such as reproduction, food intake, energy homeostasis, body temperature or hydric balance.

The hypothalamus occupies the ventral half of the diencephalon on both sides of the third ventricle and lies immediately above the pituitary gland. Dorsally, the hypothalamus is bounded by the zona incerta, and the medial edge of the cerebral peduncle corresponds to its lateral border. Caudally, the hypothalamus merges with the periaqueductal gray and ventral tegmental area of the midbrain [30].

The periventricular zone contains most of the neurons that project to the pituitary and are primarily involved in regulating secretion of hormones from this gland[30]. This zone is composed of four regions: preoptic, anterior, intermediate (or tuberal) and posterior **(Figure 2)**.

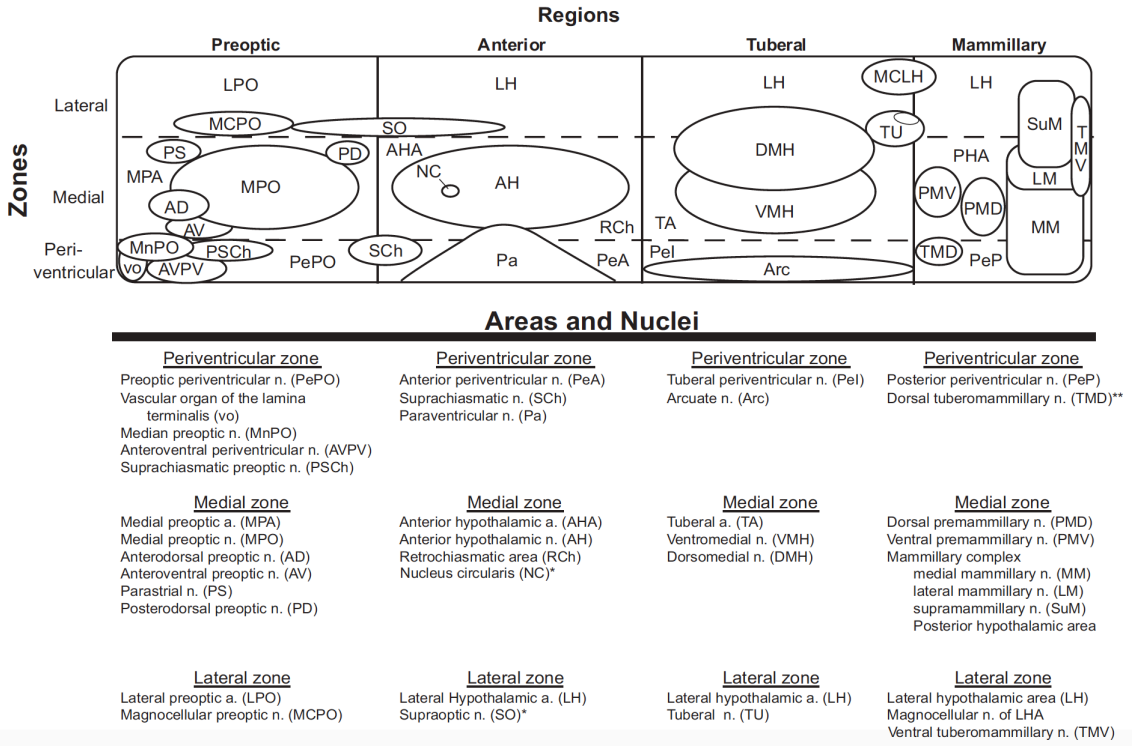


Figure 2: Schematic representation of the morphological organization of the rat hypothalamus. (Taken from *"Organization of the hypothalamus", Richard B. Simerly. The Rat Nervous System, Fourth Edition, 2015, Paxinos G.*).

Because most of the studies included in this Doctoral Thesis have been developed in rats, we will specifically describe the above hypothalamic regions in the context of this animal model.

Preoptic Region: In terms of reproductive function, one of the most relevant nuclei of this region is the anteroventral periventricular nucleus (AVPV). AVPV occupies a ventral position and is located immediately caudal to the vascular organ of the lamina terminalis (OVLT). Diferent studies support its functional role as a nodal point in neural circuitry controlling gonadotropin secretion[31,32]. AVPV neurons appear to provide direct inputs to gonadotropin releasing hormone-containing neurons in

the region adjacent to the OVLT as well as to tuberoinfundibular dopaminergic neurons in the arcuate nucleus[33]. Some of these AVPV neurons express kisspeptin, a critical neuropeptide for the neuro-endocrine control of reproduction that has been involved in the genesis of the preovulatory LH surge in this particular hypothalamic nucleus (see *"Kisspeptin"* in the *"Control of puberty"* section for further details)[5]. The heaviest projections from the AVPV are to nuclei within the periventricular zone of the hypothalamus, including the paraventricular nucleus (PVN), and these regions also regulate autonomic function[30].

Anterior Region: Two of the most recognizable hypothalamic nuclei that reside in this region are (i) the suprachiasmatic nucleus (SCh), which receives a direct input from the retina and have a critical role in the control of rodent circadian and diurnal rhythms; and (ii) the PVN nucleus, which contains neurons that express hypothalamic releasing hormones (e.g. corticotropin-releasing hormone) and project to the median eminence[30]. This hypothalamic region also contains neurons that provide direct projections to regions containing preganglionic autonomic neurons, as well as to the posterior pituitary[34,35]. Accordingly, the PVN nucleus is thought to play a central role in mediating hypothalamic responses to stress, feeding, drinking behavior and participates in a variety of autonomic responses[35,36]. Additionally, the parvicellular parts of the PVN nucleus share strong bi-directional connections with other nuclei in the periventricular zone such as the AVPV and the arcuate nucleus (ARC)[33].

Tuberal Region: The ARC is one of the most representative nuclei of this region and contains key neurons for the central control of (i) energy balance, such as the anorexigenic propiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neurons and the orexigenic neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons[37] and (ii) reproductive function, including kisspeptin neurons. It is worth to note that, unlike AVPV, ARC kisspeptin neurons seem to mediate the inhibitory effect of estrogen on gonadotropin release (see *"Kisspeptin"* in the *"Control of puberty"* section for further details)[5]. The strongest inputs to the ARC are from the PVN and the AVPV, as well as from medial preoptic nucleus, the dorsomedial nucleus and the ventral premammillary nucleus[30]. The projections of the ARC are largely confined to the periventricular zone, but notably avoid the suprachiasmatic nucleus[30].

Mammillary Region: This region is occupied solely by the caudal part of the posterior periventricular nucleus (PeP), which surrounds the posterior end of the third ventricle (3V). Because its cells resemble those of the ARC, it is often included as part of this nucleus, but on neurochemical and connectional grounds it appears to be more closely related to the periventricular nuclei [30].

GONADOTROPIN-RELEASING HORMONE (GnRH)

GnRH was isolated from porcine hypothalamus and structurally identified as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly·NH2)[38]. This decapeptide is considered as the central regulator of the reproductive hormonal cascade in a large number of species, including prominently mammals[39].

Molecular phylogeny of GnRH shows that there are three distinct forms, GnRH1, GnRH2 and GnRH3, that arose from a common origin[40]. Most vertebrate classes have only GnRH1 and GnRH2, including some teleosts, amphibians and mammals[41]. To date, GnRH3 has only been found in teleosts[41]. In mammals, hypophysiotropic functions are limited to GnRH1; therefore, we will use the terminology GnRH to refer to GnRH1 in this Doctoral Thesis.

GnRH neurons originate from the medial olfactory placode and migrate into the hypothalamus during the mid-gestational period[42]. The location of hypothalamic GnRH neurons depends on the species. In humans and nonhuman primates, a large part of hypothalamic GnRH neurons are located dorsally in the medial basal hypothalamus (MBH), the infundibulum, and periventricular region [43]. In rats, hypothalamic GnRH neurons are found in rostral areas, including the medial preoptic area, the diagonal band of Broca, the septal areas, and the anterior hypothalamus[43].

GnRH is secreted from the median eminence into the portal circulation and therein carried to the anterior pituitary[44]. Two distinct modes of GnRH secretion have been described: (i) pulsatile mode, where there are distinct pulses of GnRH secretion into the portal circulation with undetectable GnRH concentrations during inter-pulse intervals; and (ii) surge mode, which occurs in females (during the pre-ovulatory phase) and is characterized by the presence of a persistently elevated concentration of GnRH in the portal circulation[45]. Episodic secretion of GnRH, which is mandatory for proper stimulation of gonadotropin release and, hence, of gonadal function, is the result of the interplay between the intrinsic oscillatory nature of GnRH neurons and a wide array of excitatory and inhibitory afferents that integrate at the so-called GnRH pulse generator[46]. In monkeys and humans, the reduced activity of the GnRH pulse generator during the infantile-juvenile development is increased at the time of puberty[47]. Indeed, this re-emergence in the pattern of pulsatile GnRH release is considered as a hallmark of puberty onset. In female rodents, hypothalamic GnRH content increases steadily during the neonatal period. It remains constant during the first part of the infantile period, between postnatal days (PND) 8 and 12, subsequently increases during the juvenile period, and reach a maximum during the peripubertal period[48]. In male rodents, however, GnRH content does not peak but continues to increase throughout adulthood[48].

PITUITARY

The pituitary is an endocrine gland located beneath the mesodiencephalic junction and functionally connected to the hypothalamus by the median eminence via a small tubular structure called the pituitary stalk. This gland is formed by the neurohypophysis and the adenohypophysis[49].

The neurohypophysis consists of the neural lobe (pars nervosa), the pituitary stalk, and the median eminence. The neural stalk is the distal continuation of axons in the internal layer of the median eminence, which originate primarily from arginine-vasopressine (AVP)- and oxytocin (OX)-secreting magnocellular neurons of the supraoptic and PVN (**Figure 3**). Neural lobe is characterized by the terminal arborizations of these axons, a rich vasculature, and glial cells (pituicytes). Additional inputs to the neural lobe arise from brain stem catecholaminergic neurons in the nucleus of the solitary tract [50] and rostral periventricular dopamine neurons[51]. Small numbers of other neural lobe axons contain a variety of substances that may modulate hormone release at the terminal level[52].

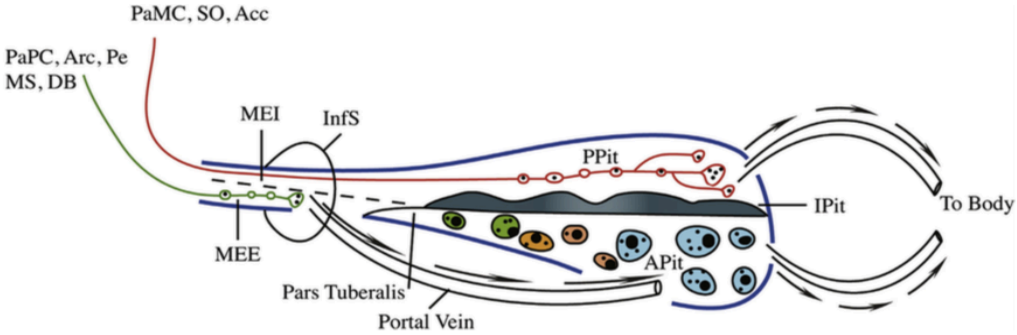


Figure 3: Midsaggital representation of the pituitary gland and its relationship with neuroendocrine hypothalamus. Magnocellular neurons of the paraventricular (PaMC), supraoptic (SO), and accessory neurosecretory (Acc) nuclei send axons (red) through the internal layer of the median eminence (MEI) and infundibular (neural) stalk (InfS) to terminate in the posterior (neural) lobe of the pituitary (PPit) and release oxytocin (OX) and vasopressin (AVP) into the general circulation. Parvocellular neurons of the paraventricular (PaPC), periventricular (Pe), arcuate (Arc), medial septum (MS), and diagonal band of Broca (DB) project axons (green) to the portal capillary plexus of the external layer of the median eminence (MEE). From here, hypothalamic releasing and inhibiting factors reach the anterior lobe (APit) via portal veins to modulate release of the APit hormones. (Taken from *"Hypothalamic Supraoptic and Paraventricular Nuclei", William E. Armstrong. The Rat Nervous System, Fourth Edition, 2015, Paxinos G.*).

The adenohypophysis consists of the anterior lobe (pars distalis), the intermediate lobe (pars intermedia), and the pars tuberalis. The anterior lobe is composed of various hormone- secreting cells and connects to the median eminence by small portal veins running along the neural stalk (**Figure 3**). These vessels carry releasing and inhibiting factors to the anterior lobe upon their secretion from hypothalamic axons into the fenestrated capillary of the median eminence, which feeds into portal veins. The intermediate lobe contains a large population of neural crest-derived cells that synthesize members of the proopiomelanocortin family of peptides (e.g. ß-endorphin and α-melanocyte-

stimulating hormone) and which receive a substantial neural innervation from dopamine, serotonin and GABA-containing axons[49].

GONADOTROPINS: LH & FSH

The gonadotropins, a family of closely related glycoprotein hormones, include luteinizing hormone (LH) and follicle stimulating-hormone (FSH), which are produced by the same pituitary cells, the gonadotrophs, and chorionic gonadotropin (CG), which is of placental origin[53].

LH is a glycoprotein dimer consisting of two subunits: α- and ß-subunits. The α-subunit is common in LH and FSH and consists of 92 amino acids. The ß-subunit of LH consists of 121 amino acids and one to two sialic acid residues, giving it a short half-life of approximately 20 min. Because of this shorter half-life, LH needs to be rapidly synthesized and typically has pulses higher in amplitude than FSH[54].

FSH is also a glycoprotein dimer consisting of two subunits: α- and ß-subunits. The ß-subunit is hormone-specific and consists of 118 amino acids and five sialic acid residues. Sialic acid residues are responsible for the half-life of the hormone, where the higher sialic acid contents the longer the half-life of that molecule. FSH has a half-life of several hours[54].

As previously described for GnRH, there are also two modes of secretion for LH and FSH, the tonic (or basal) and surge. The tonic mode accounts for the secretion of these hormones in the male, and for the low circulating LH and FSH levels observed in females during the greater part of the ovarian cycle[47]. In the male, this mode of secretion is responsible for maintaining spermatogenesis and stimulating testosterone secretion (*see "Gonads"* section). In the female, this mode drives folliculogenesis and estradiol production in the follicular phase and maintains the corpus luteum in the second half of the cycle (*see "Gonads"* section). The surge mode of gonadotropin secretion triggers ovulation, and is termed the preovulatory gonadotropin surge[47]. The transition into puberty in both boys and girls is tightly coupled with an initiation of a progressive increase in the secretory activity of the pituitary gonadotropes, as reflected by the temporal changes in the profiles of circulating LH and FSH concentrations during this phase of human development. The increase in LH at this stage of development is particularly robust[47].

In female rodents, circulating FSH levels rise dramatically and reach peak values at the beginning of the infantile period (PND12). This infantile FSH surge enhances the development of preantral follicles and rescues early antral follicles from apoptotic death (*see "Gonads"* section). LH levels are also elevated and sporadic bursts of secretion have been reported to occur[48]. By the end of the infantile period, circulating FSH levels decrease to nadir values, the sporadic bursts of LH release disappear, and plasma LH levels remain low[48]. The transition between the juvenile and the peri-pubertal periods is marked by the appearance of morning–afternoon differences in the serum LH

concentrations. This diurnal pattern of release is established in female rats around PND30 and is characterized by an increase in both basal LH levels and LH pulse amplitude in the afternoon[48].

In male rodents, circulating FSH levels rise dramatically after the second week of life and reach a maximum between PND 30 and 40 days, then they decrease when serum testosterone concentrations attain levels similar to those seen in adults[48]. Changes in gonadotropin secretion precede the maturation of the testes. While FSH binds to Sertoli cells and influences spermatogenesis, LH stimulates testosterone secretion by acting directly on the Leydig cells (*see "Gonads"* section)[48].

GONADS

The gonads include the testes in males and the ovaries in females and play a dual role in both sexes, as they are responsible for (i) generating and releasing mature gametes from germ cells (gametogenesis), and (ii) synthesis and secretion of steroid and peptides hormones (hormonogenesis) that regulate, among other actions, the production of these gametes.

Testis

The mammalian testes are paired oval structures that produce the male gametes and the male sexual hormones (mainly, androgens). The term spermatogenesis describes and includes all the processes involved in the production of gametes, whereas steroidogenesis refers to the enzymatic reactions leading to the production of male steroid hormones. Spermatogenesis and steroidogenesis take place in two different compartments: the *tubular compartment* (seminiferous tubules) and the *interstitial compartment*, which are closely connected to each other. The function of the testis and its compartments are subjected to neuroendocrine regulation by the hypothalamus and the pituitary gland[55].

The *interstitial compartment* contains Leydig cells, immune cells, blood and lymph vessels, nerves, fibroblasts and loose connective tissue. Leydig cells are the most important component of this compartment as they produce and secrete testosterone (T). Two different populations of Leydig cells have been described in most mammalian species, including humans and rodents: fetal- and adult-type Leydig cells[56]. Of note, the fetal Leydig cells are replaced by the adult population during the prepubertal period[56]. The testes of post-infantile, prepubertal macaques and humans contain few recognizable Leydig cells and secrete low levels of testosterone[47]. The development of Leydig cells and a rise in intratesticular androgen concentration likely precede both the acceleration in testicular growth and the rise in circulating androgen levels. In humans, daytime levels of plasma testosterone begin to rise during the tenth year of life and continue to increase progressively before reaching adult values at 14–15 years of age[47].

Spermatogenesis takes place in the *tubular compartment*. This compartment contains the germ cells and two different types of somatic cells, the peritubular cells and the Sertoli cells. Sertoli cells are somatic cells located within the germinal epithelium that synthesize and secrete a large variety of factors: proteins, cytokines, growth factors, opioids, steroids, prostaglandins, modulators of cell division, etc.[55]. In contrast to the steroidogenic component of the primate testis, which appears to be quiescent during the postinfantile and prepubertal stage of development, the seminiferous cords lengthen during this phase of development due to proliferation of Sertoli cells and undifferentiated type A spermatogonia[47]. The acceleration in testicular growth during early puberty results largely from an increase of the seminiferous tubules associated with the appearance of mature Sertoli cells and proliferation of germ cells. The generation of sperm via spermatogenesis is a continuous process throughout the reproductive lifetime. In mature testes, spermatogenesis progresses in a well-coordinated manner, known as the spermatogenic wave[57].

In rodents, different studies have shown that changes in the secretion of gonadotropins precede the maturation of the testes. FSH binds to Sertoli cells within the seminiferous tubules to promote their proliferation, which quantitatively influences spermatogenesis, but might not be absolutely indispensable to reach fertility[48]. LH stimulates testosterone secretion by acting directly on the (adult type) Leydig cells that develop postnatally (around PND7 in mice) and whose proliferation and differentiation are also highly dependent on LH[48]. Testosterone promotes spermatogenesis, potentiates the pituitary response to GnRH and exerts a negative feedback effect on the hypothalamic–pituitary axis throughout life, even during the neonatal period[48].

Mature germ cells (spermatozoa) are first observed around 40 and 55 days of age in mice and rats, respectively, when the incidence of fertile matings increases. These events occur concomitantly with an increase in testosterone levels, whose pattern of secretion is highly correlated with pulses of LH secretion[48]. The complete initiation of spermatogenesis takes around 40 and 54 days in mice and rats, respectively, a time period that corresponds to the age at puberty in these species[48].

Ovary

The ovary consists of an outer cortex and an inner medulla. The ovarian follicles are found mainly in the cortex, while the medulla mainly contains fibromuscular tissue and vasculature. Each ovarian follicle consists of an oocyte surrounded by layers of and theca cells. The follicles are present in the cortex in a wide range of sizes representing various stages of folliculogenesis[54] **(Figure 4)**. The first level of follicular organization is the *primordial follicle*, which is formed by a primary oocyte surrounded by a monolayer of folicullar cells called granulosa cells. This primordial follicle undergoes a series of successive transformations leading to the formation of *primary follicles*, where the oocyte increases in size and begins to accumulate polysaccharides between the oocyte and granulosa cells. *Secondary follicles* are characterized by an increase of granulosa cells, which will determine the

production of the primary female estrogen, 17 ß-estradiol (E2), and the recruitment of interstitial cells that will originate the theca interna. *Tertiary or antral follicles* are characterized by an increase of granulosa cell layers and the recruitment of additional interstitial cells that form a layer surrounding the theca interna, leading to the theca externa and the formation of follicular antrum. The *preovulatory follicle or de Graaf follicle*, after LH-mediated follicular rupture, allows the exit of the secondary oocyte from the ovary at ovulation. Earlier stages of follicular development are independent of central nervous system hormone production, while later stages of follicular development will depend on reproductive hormones produced by the anterior pituitary. The growing ovarian follicle will produce estradiol from the granulosa cells. After ovulation, the remnant cells of the follicle luteinize and start secreting progesterone. The granulosa cells are also responsible for the secretion of inhibin as well as anti-Müllerian hormone (AMH)[54].

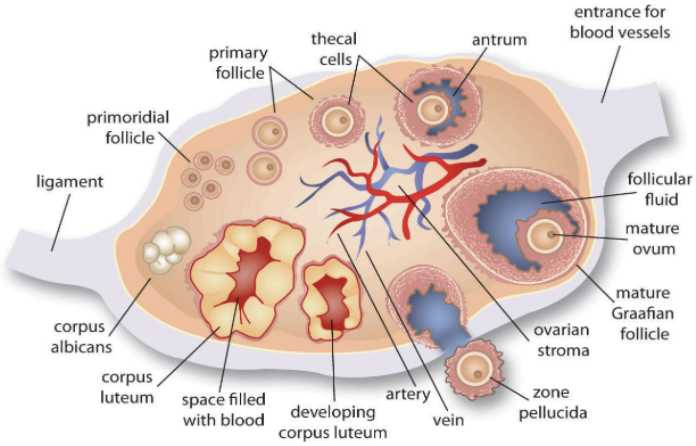


Figure 4: Diagram showing anatomical structure of the ovary. The ovarian cycle consists of the follicular phase, ovulation, and luteal phase (Taken from "*Structure Of Ovary Diagram*" Anatomysciences.com).

In primates and humans, growth and atresia of ovarian follicles begin in utero and continue during the prepubertal years. Graafian (preovulatory) follicles are not observed during prepubertal development. Whereas primordial and pre-antral follicles predominate during the prepubertal years, small antral follicles can develop prior to puberty[47]. The prepubertal ovary actively secretes estradiol. Cross-sectional studies of girls have demonstrated that between 8 and 10 years of age, estradiol serum concentrations begin to increase to values typically seen in adult women during the early follicular phase of the menstrual cycle[58,59]. Concomitant with the rising estradiol concentrations the initiation of breast development occurs. In the human female, further development of secondary sexual characteristics occurs as puberty progresses and culminates in menarche (first menstruation), which generally occurs 2–3 years following the onset of breast development[47].

In female rodents, primordial follicles are formed by 3 days after birth, whereas well-developed secondary follicles are seen by PND7[48]. During the second and third weeks of life, when the first wave of ovarian follicles have reached the secondary stage and become responsive to FSH, key

neuroendocrine events prompt them to develop into preantral and then antral follicles over the next 3 weeks[48]. An infantile surge in FSH enhances the development of preantral follicles and rescues early antral follicles from apoptotic death[60]. LH does not appear to play a major role in ovarian development at this early stage of sexual maturation, since LH receptor knockout mice, in contrast to FSH knockout animals, exhibit early antral follicles[61]. Between PND7 and 21, there is an overall increase in steroidogenic enzyme activity in the ovary that leads to a marked increase in the production of estrogens. However, the negative feedback of ovarian estradiol onto the hypothalamus is relatively ineffective during the infantile period[48]. During the juvenile period, the hypothalamic–pituitary axis becomes sensitive to the low levels of estradiol produced by the ovaries, thus enabling the development of negative feedback by estradiol. In parallel, the hypothalamic–pituitary axis acquires the ability to respond to high exogenous estrogen levels, although the ovarian follicles at this stage of development do not produce sufficient amounts of estradiol to exert a positive feedback effect and stimulate a surge of LH[48]. The acquisition by the ovary of the capacity to secrete high levels of estrogens over a period of about 24h represents the key event in the determination of the timing of the first preovulatory GnRH/LH/FSH surge and the onset of puberty[62,63]. Female rats show cyclic variations in their reproductive activity every 4-5 days, named estrous cycle (**Figure 5**).

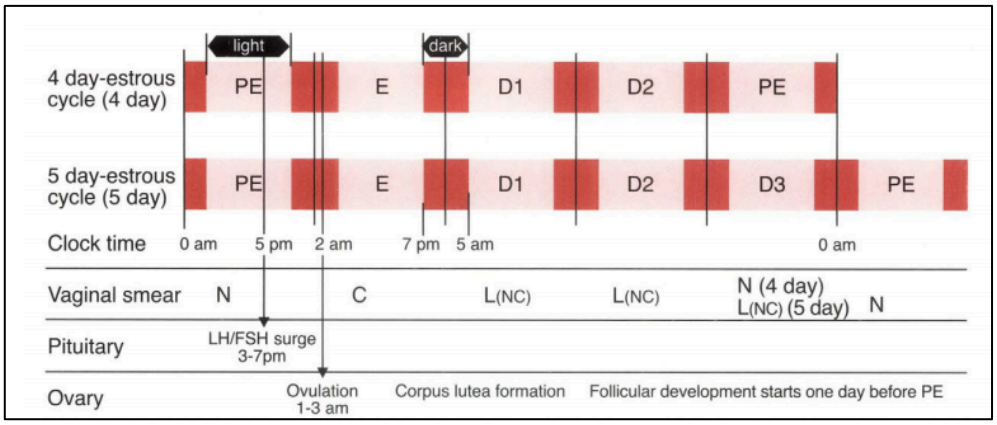


Figure 5: Schematic representation showing the 4- or 5-day rat estrous cycle (upper panel) and cell populations in the vaginal smears as well as physiological events during the cycle (lower panel). PE: proestrus; E: estrus; D1: diestrus 1; D2: diestrus 2; D3: diestrus 3; N: nucleated cell; C: cornified cell; L: leukocyte. (Taken from *"Physiology of Reproduction", K. Maeda et al. The Laboratory Rat, Fourth Edition, 2015, Krinke, G.J.*).

An estrous cycle consists of proestrus, estrus and diestrus. A 4-day cycle has a 2-day diestrus (diestrus 1 and 2) and a 5-day cycle has a 3-day diestrus (diestrus 1, 2 and 3). Diestrus 1 is often called metestrus. Vaginal smears are widely used to identify the phase of the estrous cycle and the observation of certain cell populations in vaginal smears is the most popular and reliable method for identifying the different phases. Nucleated cells are the major population in the smear at proestrus. At estrous, the vaginal smear is full of markedly swollen cornified cells lacking nuclei. At diestrus 1 and 2, numerous leukocytes appear in addition to the nucleated cells and shrinking cornified cells [64].

In addition to the classical endocrine control, increasing evidence supports that the ovarian function is also subjected to neural regulation[46,65]. Using a viral transneuronal tracing technique, Gerendai et al. demonstrated that local administration of a viral tracer into the ovary produced intense cell-body labelling in the PVN[66]. This nucleus represents the specific forebrain region from which cells project to preganglionic sympathetic neurons. These sympathetic neuronal inputs impinge on the celiac postganglionic cells, whose efferents innervate the ovary[67] via two different routes: (i) the ovarian nerve plexus (ONP), mostly responsible for the perivascular innervation of the ovary, and (ii) the superior ovarian nerve (SON), which specifically innervates the secretory elements of the ovary, such as follicles and interstitial glands[67] (**Figure 6**).

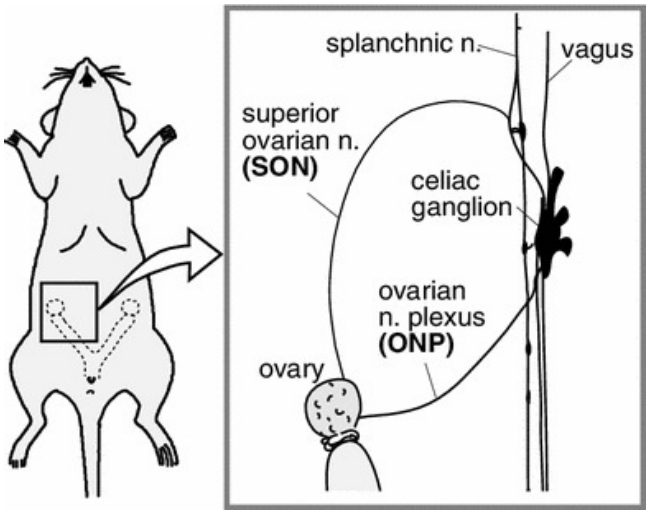


Figure 6: Schematic illustration of ovarian innervation by the ovarian nerve plexus (ONP) and the superior ovarian nerve (SON). (Taken from *"Autonomic nervous regulation of ovarian function by noxious somatic afferent stimulation", S. Uchida and F. Kagitani. J. Physiol. Sci, 2015.*).

The sympathetic innervation of the ovary has been involved in relevant aspects of ovarian function, including steroidogenesis and early follicular development[46,68]. These actions are performed mainly by norepinephrine (NE) and the vasoactive intestinal peptide (VIP). While NE stimulates ovarian production of progesterone and androgens (but not estradiol) via activation of β2-adrenergic receptors, VIP induces progesterone, estradiol and androgen secretion[69,70]. Interestingly, two pieces of evidence strongly support the existence of an enhaced ovarian sympathetic activity by the time of ovulation: (i) a rise in ovarian NE secretion at the time of the preovulatory surge of gonadotropins[71] and (ii) a greater increase in ^{3}H-NE release during the transition proestrus-estrus at ovarian level[72]. Of note, surgical or pharmacological removal of the extrinsic ovarian innervation has been shown to reduce the number of antral follicles[73]. Moreover, immunosympathectomy during the neonatal period evokes a permanent loss of the sympathetic innervation in female rats; a phenomenon that has been linked to delayed follicular development, reduced ovarian steroid output, and marked irregularities of estrous cyclicity[74,75].

In addition to the extrinsic innervation, the ovary also contains an intrinsic innervation. This innervation was first inferred by experiments showing that when the ovary is transplanted into an ectopic site, reinnervation occurs promptly[76]. This phenomenon is mostly due to the presence of a family of growth factors, known as neurotrophins (NTs), in the ovary[77]. Among them, the nerve growth factor (NGF) and its receptors, tyrosine receptor kinase A (trkA), also known as tropomyosin receptor kinase, and the low-affinity neurotrophin receptor p75 (p75 NTR), seem to play a prominent role.

Indeed, the immunoneutralization of NGF actions during the early postnatal period has been shown to deprive the ovary from sympathetic nerves and prevents its reinnervation during adulthood[76]. Additionally, NGF appears to stimulate ovarian development and contributes to the acquisition of a mature ovarian function by facilitating antral follicular growth and promoting the acquisition of gonadotropin receptors and responsiveness to gonadotropins in the developing follicles[77].

3. CONTROL OF PUBERTY

CENTRAL REGULATORS

As previously mentioned, the initiation of puberty in mammals requires a sustained increase in the neurosecretory activity of GnRH neurons[78,79]. This increase is determined by coordinated changes in transsynaptic and glial inputs to the GnRH neuronal network, consisting of an increase in stimulatory signals and the loss of inhibitory influences[78,79].

A relevant part of the excitatory control of puberty is provided by neurons that synthesize glutamate[80], kisspeptins[81], and neurokinin B (NKB)[82] as neuronal transmitters. Of note, kisspeptins, the peptide products encoded by the *Kiss1* gene, and NKB, a tachykinin peptide encoded by *TAC3/Tac2* gene, are coexpressed in the same cell type in the ARC, which has been termed KNDy neurons[83]. Apparently, the release of NKB from KNDy neurons can activate kisspeptin secretion from the same cell type and, eventually, modulate GnRH neurosecretory activity[84,85].

The inhibitory circuitry responsible for the control of puberty is largely dependent on neurons that produce GABA, endogenous opioids, and eventually RFamide-related peptides[86]. GABAergic neurons can modulate GnRH release through indirect actions on neurons connected to the GnRH neuronal network[78], or direct actions mediated by the activation of GABA-A receptors on GnRH neurons[87,88]. Opiatergic neurons inhibit GnRH release through different peptides. Among them, dynorphin (Dyn), encoded by prodynorphin (*Pdyn*) gene, seems to have a relevant inhibitory role. In particular, the release of dynorphin from KNDy neurons has shown to inhibit pulsatile GnRH secretion by acting upon κ-opioid receptors (KOR)[83]. Because KOR has been detected in both KNDy and GnRH neurons, the inhibitory effects of dynorphin on pulsatile GnRH secretion may occur either indirectly or directly [89]. RFRP neurons may produce one or two peptides, RFRP1 and RFRP3, acting on a single receptor expressed in GnRH neurons, NPFFR1, to directly inhibit its neurosecretory activity[90,91]. In addition to neuronal inputs, glial cells, such as astrocytes, participate in the regulation of GnRH neurons and puberty via two mechanisms: the release of growth factors and other bioactive molecules, and plastic changes in glial-to-GnRH neuron contacts and adhesiveness[79].

Because kisspeptins have been shown to be especially relevant in the central control of puberty and their roles have been partially explored in the context of the present Doctoral Thesis, we will

describe some of the major features of these intriguing neuropeptides in the context of puberty onset.

Kisspeptins

Kisspeptins are a family of structurally related peptides of different amino acid length (e.g., Kp-54 and Kp-10) that share the C-terminal region. Kisspeptins are encoded by the Kiss1 gene and operate through the G protein-coupled receptor, Gpr54, which is also termed kisspeptin receptor or Kiss1R [5,92]. The reproductive roles of kisspeptins and GPR54 were disclosed in 2003, when inactivating mutations of GPR54 were described in patients with hypogonadotropic hypogonadism (HH); a condition of impuberism and infertility of central origin[93,94]. Mice engineered to lack functional GPR54 (or KISS1) were shown to be a complete phenocopy of affected humans[93]. Departing from those initial observations, different studies have addressed the putative physiological roles of kisspeptin signaling in the timing of puberty. These studies have documented a complex and multifaceted pattern of activation of the Kiss1 system during the pubertal transition, which seems to involve, at least, the following major components: (i) an increase in endogenous kisspeptin tone, sufficient per se to fully activate the GnRH/gonadotropin axis; (ii) an enhancement of GPR54 signaling efficiency; (iii) an elevation in sensitivity to the stimulatory effects of kisspeptins; and (iv) an increase in the number of kisspeptin neurons and their projections to GnRH neurons[5] (**Figure 7**).

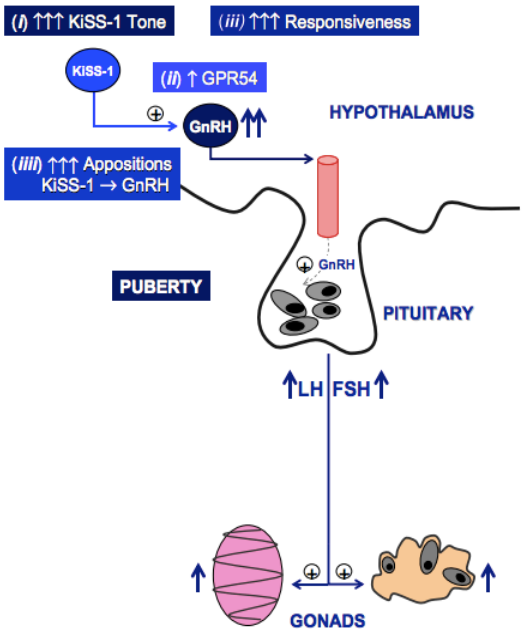


Figure 7: Tentative model for the potential mechanisms whereby Kiss1 system contributes to the activation of the HPG axis at puberty. (Taken from *"New frontiers in kisspeptin/GPR54 physiology as fundamental gatekeepers of reproductive function", Juan Roa et. al. Frontiers in Neuroendocrinology, 2008*).

The relevance of proper kisspeptin signaling for the precise timing of puberty is reinforced by the observation that pharmacological blockade of Gpr54 delays puberty onset in rats[95] and timed

ablation of Kiss1 neurons in the juvenile period prevents the attainment of reproductive competence[96]. Furthermore, the infertility linked to the selective deletion of Gpr54 expression in GnRH neurons and the rescue of fertility induced by the reintroduction of GPR54 into these neurons in Gpr54 knockout mice emphasize the essential role of GPR54 expression in GnRH neurons for the onset of puberty and reproduction[97]. Interestingly, one study suggested a dispensable role of kisspeptin signaling in puberty onset in rodents, as it showed that congenital ablation of Kiss1 neurons in female mice was not incompatible with the attainment of adult fertility[96]. However, this phenotype could be due to incomplete neuronal ablation and/or developmental compensation. Indeed, in the same study, it was demonstrated that timed elimination of Kiss1 neurons during the early juvenile period prevented normal pubertal maturation and caused infertility[96]. These findings further document the complex pattern of developmental regulation of Kiss1 neurons that, despite potential compensatory mechanism, are essential for the timed activation of the HPG axis that leads to puberty.

Detailed neuroanatomical studies in rodents have identified two prominent populations of kisspeptin neurons in the hypothalamus, one located in the ARC and the other in the AVPV. These two populations are sexually dimorphic and functionally different. Thus, while kisspeptin neurons are present in the ARC of both sexes[98], a greater density has been observed in females than in males in the AVPV[99]. Furthermore, AVPV kisspeptin neurons seem to be involved in the generation of preovulatory GnRH/LH surge through positive feedback mechanisms, whereas ARC kisspeptin neurons have been proposed to mediate the negative feedback effect of sex steroids upon GnRH release[100]. This is based on the ability of those hypothalamic populations to respond to estrogen in a totally opposite manner. Thus, while AVPV kisspeptin neurons have been shown to respond to estrogen with increased synthesis of kisspeptin, the production of kisspeptin is inhibited by estrogen in the ARC[100,101].

Whether the activational program described above applies equally to these hypothalamic populations of Kiss1 neurons is yet to be characterized. Interestingly, mouse studies have suggested that estrogens are indispensable for the pubertal expansion, and possibly activation, of Kiss1 neurons, mainly in the AVPV. This would imply that the initial stages of puberty, and hence the early rise of circulating sex steroids, would take place independently of the kisspeptin drive. Thus, rather than the trigger of puberty, the Kiss1 system would operate as an indispensable amplifier of the secretory activity of GnRH neurons during puberty[102]. In any event, it must be stressed that equivalent studies in other species have not been conducted to date. In this context, it is worthy to note that the increase of the pulsatile secretion of gonadotropins in primates can take place in the absence of gonadal function; a finding that may argue against an indispensable role of sex steroids in the pubertal activation of the Kiss1 system in primate species. Of important note, a novel view of the role of kisspeptin neurons in the central control of puberty onset has recently emerged. In particular, it has

been proposed that ARC kisspeptin neurons might act as an integral component of the hypothalamic GnRH pulse generating mechanism responsible for the increase of GnRH secretion at the time of puberty onset[41,103]. According to this notion, ARC kisspeptin neurons would be a relevant element of the upstream regulatory mechanisms determining the timing of puberty onset[103]. Furthermore, using an optogenetic approach, it has demonstrated that ARC kisspeptin neurons can synchronize their activity and stimulate GnRH neurons by activating AVPV kisspeptin neurons[104].

ENVIRONMENTAL FACTORS

Today, it is well known that the development of the above hypothalamic GnRH network depends on environmental factors, which are not only crucial for puberty to occur, but also for its timing. Interestingly, while genetic determination plays a relevant role in the pubertal process[105], the substantial variation in the age of puberty detected even within homogeneous populations points to environmental cues as key determining factors for the tempo of puberty [1]. In this sense, recent epidemiological studies have documented a trend for an earlier initiation of puberty in girls, as estimated by the beginning of breast development[106,107], and boys, as estimated by the beginning of genital and pubic hair growth[108]. This phenomenon seems to be related to the increased exposure to compounds with sex steroid activity (e.g. endocrine-disrupting compounds, EDCs) and the higher prevalence of childhood obesity[106,109,110].

Sex steroid environment

The attainment of reproductive capacity at puberty depends on the proper functional organization at early stages of development of the central (hypothalamic) circuitries responsible for the control of the pulsatile secretion of GnRH. Probably, one of the major events in the development of those hypothalamic networks is the process of sexual differentiation of the brain[111,112]. Although sex chromosome (and somatic) genes play a key role, sex steroids seem to be primarily responsible for this phenomenon to occur[112]. This contention was initially suggested by Phoenix et al., who demonstrated that the expression of male and female sexual behavior in adulthood could be masculinized and defeminized by modifying testosterone exposure during prenatal development[113]. This seminal observation suggesting that perinatal testosterone, but not ovarian hormones, drives the process of sexual dimorphism of the nervous system has been challenged and modified in recent years. Thus, recent evidence supports that ovarian hormones play an essential role in both masculinization and feminization of the brain and this phenomenon is not just limited to perinatal stages, but also includes the pubertal period[114].

Rodent data have firmly demonstrated that, while *brain masculinization* is primarily determined by estradiol, which is locally produced at high levels by aromatization of testis-derived testosterone, *brain feminization* requires low estrogen input (due to quiescent ovaries) and high levels of circulating α-fetoprotein[111]. Importantly, such early differentiation phenomena translate into relevant

sexually dimorphic features, such as the timing of puberty[78], as well as different behaviors and neuroendocrine secretory patterns later on life[111]. A hallmark of such neurohormonal sexual dimorphism is the cyclic secretory activity of the GnRH/gonadotropin system, which is based on the capacity of estrogens, selectively in the female, to induce the preovulatory surge of gonadotropins from puberty onwards[111].

Recent evidence has also suggested that, in addition to early (perinatal) periods, sex steroid input might induce permanent functional alterations of different neurohormonal axes during the peripubertal period[115]. Indeed, important developmental effects of estrogen on key hypothalamic networks governing puberty onset have been recently documented during (pre)pubertal maturation in mice[27,116]. In addition to such potential organizing effects, it is well known that the pubertal transition is characterized by substantial acute changes in the sensitivity and responsiveness of reproductive hypothalamic networks to the regulatory effects of sex steroids[117]. These changes importantly contribute, together with gonadal-independent variations of the central excitatory and inhibitory inputs to GnRH neurons, to define the timing of puberty.

Metabolic factors

Another key component for the proper development of reproductive function is the nutritional environment. In this sense, it is well known that metabolic conditions and the magnitude of body fuel reserves play a key role in the regulation of puberty[118]. This phenomenon enables that reproductive capacity is acquired only if proper fuel stores and metabolic conditions are attained[118,119]. The influence of metabolic signals on puberty and fertility is not only evident in the female, but also in the male, in which energy requirements for reproduction are also evolutionary important, e.g. for territoriality and partner selection. Moreover, not only conditions of energy deficit, such as anorexia or strenuous exercise, but also situations of persistent energy excess, such as morbid obesity, are associated with pubertal alterations[112]. For instance, early forms of nutritional or metabolic challenge, such as under- or over-nutrition taking place during the fetal, neonatal and prepubertal life, have been shown to alter the timing of puberty in rodents[112]. In the same line, several epidemiological studies have suggested that the recent decline in the age of puberty observed in girls might be related to a higher prevalence of childhood obesity[106,107]; a phenomenon that has caused considerable concerns among scientists and even the lay public mainly due to the potential adverse health outcomes of early puberty, which include increased risks of disordered behavior, cardiovascular and metabolic disease, hypertension, obesity, and diabetes, as well as lower adult height and even reduced life expectancy[120-124].

During the last two decades, considerable progress has been made towards the elucidation of the mechanisms and signals responsible for the alterations in the timing of puberty associated with conditions of metabolic stress. A major breakthrough in this front was the identification of the adipose

hormone, leptin, as an essential neuroendocrine integrator responsible for coupling of the state of body energy stores and different hormonal functions, including reproduction[6,125]. In addition to leptin, other peripheral hormones from key metabolic tissues, such as the gastrointestinal tract, the pancreas and the adipose, have been proposed as putative regulators of the gonadotropic axis[126]. Prominent among them is the gut hormone, ghrelin, which operates as a signal of energy insufficiency and functional antagonist of leptin[127]. Indeed, experimental evidence accumulated in recent years strongly suggests its physiological, and eventual pathophysiological role in the regulation of puberty onset and gonadal function[127].

Leptin: The adipose hormone, leptin, is released to the circulatory system in proportion to the size of fat stores[128] and acts as an anorexigenic and thermogenic factor within the hypothalamus to adjust energy requirements, fat reserves, and food intake[125]. Nowadays, leptin is recognized as an indispensable factor in the metabolic control of puberty and fertility[3]. This is clearly illustrated by the negative impact of conditions of leptin insufficiency on reproductive maturation and function, which are often linked to delay or absence of puberty and compromised fertility. This phenomenon has been observed both in different experimental models of leptin deficiency, including genetically-altered animal models[129,130] and early undernourished rodents[6,131], as well as in human pathologies associated with low or null leptin levels[129,132].

The first studies that addressed the functional role of leptin in the control of puberty suggested that leptin might act as a primary stimulatory signal of the reproductive system. Thus, Ahima et al. showed that chronic administration of leptin at concentrations that do not alter body weight was able to induce an advance in the onset of puberty in prepubertal female mice[133]. In the same vein, immature female rats chronically treated with recombinant ovine leptin showed a significant advance in the onset of puberty; a phenomenon that was associated with increased expression of ovarian steroidogenic enzymes and gonadotropin secretion[134]. Furthermore, transgenic skinny mice with increased circulating levels of leptin displayed earlier vaginal opening and greater uterine weights than their respective controls[135]. In the same line, hyper-leptinemic female rodents resulting from early overfeeding showed, in the majority of cases, precocious puberty[6,131].

Further studies, however, suggested a predominant permissive role of leptin in the control of puberty onset. Thus, chronic leptin administration prevented the delayed puberty observed in prepubertal female rats subjected to severe food-restriction (80%)[136]; a phenomenon that was later confirmed in female mice[137]. Similarly, a different study conducted in immature female rats showed that maintenance of adequate leptin levels prevented pubertal delay in conditions of energy insufficiency[138]. Of note, similar findings were also observed in obese children congenitally deficient in leptin, where subcutaneous injections of recombinant human leptin were able to facilitate pubertal development[132]. Overall, these findings settled the contention that leptin plays a permissive, rather than a triggering, role in the metabolic control of puberty and fertility[6,129,139].

Additionally, several studies have shown that serum leptin levels change during the juvenile-pubertal transition. Thus, circulating levels of leptin significantly increase throughout puberty in girls[140]. In the same vein, a nocturnal increase in serum leptin levels has been detected in prepubertal female rats[141]. Whether proper levels of leptin are the one and only metabolic gate for puberty to proceed is yet to be solved.

Ghrelin: The gastrointestinal hormone, ghrelin, act as orexigenic factor in the central control of appetite and metabolism[142]. Ghrelin is released to the circulatory system in two major forms: acyl-ghrelin, which is able to activate the growth hormone secretagogue receptor 1a (GHS-R1a), and unacyl-ghrelin (UAG), whose receptor has not yet been clarified[143]. Acyl-ghrelin, which will be referred herein as ghrelin, results from the addition of a medium-chain fatty acid by the enzyme ghrelin O-acyl transferase (GOAT)[144,145]. This acylation reaction is indispensable for ghrelin binding to GHS-R1a and, ultimately, for exerting many of its biological functions[146]. Together, ghrelin, GHS-R1a and GOAT form the so-called ghrelin system. The concurrent actions of ghrelin upon different neuroendocrine axes[143], along with its reciprocal interactions with leptin in the control of energy balance (ghrelin as long-term signal for energy insufficiency vs. leptin as long-term signal for energy sufficiency)[147], make this gut hormone a suitable candidate for the integral control of energy balance and other essential endocrine functions, including reproduction. Nowdays, ghrelin is considered a relevant signal in the metabolic control of puberty and reproduction; its actions being predominantly inhibitory and opposite to those of leptin (which are permissive and stimulatory)[127]. Thus, chronic administration of both low (0.5 nmol/every 12 h) and high (1.0 nmol/every 12 h) doses of ghrelin to prepubertal male rats reduced serum LH and testosterone levels, and partially delayed balanopreputial separation, considered as an external index of puberty onset in males[127,148]. Of note, chronic treatment of peripubertal female rats with an analogous protocol of low doses of ghrelin (0.5 nmol/12 h for 10 days) did not cause significant changes in serum levels of gonadotropins or estradiol, neither did it alter the timing of puberty[148]. In contrast, twice daily injection of 1 nmol ghrelin for 10 days was sufficient to delay the age of vaginal opening and ovarian follicular development and ovulation in pubertal female rats[2]. As a whole, these findings suggest a putative inhibitory role of increased ghrelin levels on pubertal timing in both male and female rodents; yet, females seem to be less sensitive than males to the regulatory actions of ghrelin according to these studies. Interestingly, a recent study have documented a prepubertal increase of the hypothalamic expression of ghrelin in early under-nourished rats with delayed vaginal opening[149]; an interesting finding that provides additional support to its inhibitory role in the control of puberty onset.

The impact of ghrelin on human puberty is yet to be defined; yet, the decline in circulating ghrelin levels documented in both boys and girls during pubertal transition may suggest its potential inhibitory role in this context[150,151].

CENTRAL INTEGRATION OF THE METABOLIC CONTROL OF PUBERTY

As we previously mentioned, GnRH neurons are the final common pathway through which the brain regulates the onset of puberty. However, these neurons do not seem to be direct targets for relevant metabolic signals involved in the regulation of puberty, such as leptin or ghrelin[152]. The indirect mode of action of these hormones on the activity of GnRH neurons is suggested by the following pieces of evidence: (i) GnRH neurons show low or negligible expression levels of functional leptin receptors (LepRs)[153-155] (whether GnRH neurons express the ghrelin receptor GHS-R1a is still unknown); (ii) the icv injection of leptin in both male and female rats failed to stimulate the expression of the leptin-induced signal transducer and activator of transcription (STAT-3) in GnRH neurons[155]; (iii) the selective ablation of LepRs from GnRH neurons do not have any impact on either the timing of puberty or adult reproductive function in female mice[155]; and (iv) relevant hypothalamic neuropeptides, such as corticotropin-releasing hormone (CRH), NPY and kisspeptins, have been suggested as potential mediators for the reproductive actions of ghrelin on GnRH neurons[3]. Overall, these results point out the existence of indirect pathways, sensitive to these key pubertal regulators, which would operate as conduits for the transmission of such metabolic information to GnRH neurons.

Kisspeptins as central processors for the metabolic control of puberty

Neurons that contain receptors for (or are sensitive to) the above metabolic hormones and send afferent inputs to GnRH neurons are likely to be responsible for sensing the metabolic milieu and controlling GnRH secretion. The candidates for serving this integrative function include neurons expressing galanin-like peptide (GALP), NPY, POMC and its related products (e.g. **α**-MSH- and **β**-endorphin) in the ARC, neurons expressing orexins in the LHA, neurons expressing melanin-concentrating (MCH) in the LHA and zona incerta, neurons that produce nesfatin-1 in the LHA, PVN and SON, and kisspeptin-synthesizing neurons in the AVPV and the ARC[152,156]. Among them, kisspeptins have a prominent place. This is illustrated by two major lines of evidence:

1. The hypothalamic expression of Kiss1/Kisspeptin is altered in conditions of reproductive impairment linked to metabolic stress: Different studies have reported that extreme conditions of negative energy balance linked to perturbed reproductive maturation and function induce a suppression of the hypothalamic Kiss1 system. Thus, pubertal female rats subjected to fasting conditions showed a significant reduction in the hypothalamic expression of Kiss1, at both mRNA and protein levels; a phenomenon that was associated with low circulating levels of LH[157,158]. Similarly, female rats subjected to chronic food restriction throughout puberty (20% for 7 days) displayed a significant suppression of Kiss1 mRNA expression at the ARC[159]. Furthermore, a significant decrease in the hypothalamic levels of Kiss1 mRNA was detected in juvenile female rats born from dams subjected to 50% food restriction during pregnancy[160]. In the same vein, early undernutrition by means of rearing

in large litters (LL; 20 pups per dam), resulted in delayed puberty and a concomitant decrease in both the hypothalamic Kiss1 mRNA levels and the number of Kiss1 neurons in the ARC of female pubertal rats[161], as well as a decrease in the density of kisspeptin fibers in female mice[162].

Interestingly, several studies have shown that extreme conditions of energy excess are also able to induce significant changes in the hypothalamic expression of Kiss1 and the timing of puberty. For instance, our group reported a significant elevation in the hypothalamic Kiss1 mRNA levels and a trend for increased kisspeptin fibers in the periventricular area of early overfed female rats (reared in small litters, SL; 4 pups per dam) with precocious puberty[161]. In contrast, Smith & Spencer failed to detect these alterations at the age of VO by using a similar animal model (SL)[163]. In the same vein, exposure to high fat diet (HFD) during the pre-weaning period did not affect either the timing of puberty or the expression of hypothalamic Kiss1 mRNA in peri-pubertal female rats[164]; yet, a recent study has shown a significant increase in the number of Kiss1-expressing neurons at the ARC in female rats subjected to a similar nutritional manipulation (maternal HFD). Furthermore, HFD administration after weaning, between postnatal days 21 and 36, evoked an earlier onset of puberty and increased the frequency of pulsatile LH secretion, which was associated with elevated expression levels of Kiss1 in the medial POA and ARC of female rats during the pubertal transition[165]. Of note, however, an independent study involving HFD administration between postnatal days 21 and 34 failed to detect significant changes in the age of VO or hypothalamic Kiss1 expression in pubertal female rats[164]. Overall, these findings suggest that the hypothalamic expression of Kiss1/kisspeptin and the pubertal phenotype obtained largely depends on the degree and duration of obesity, as well as the type/features of the nutritional challenge (e.g. type of diet).

2. The normalization of the hypothalamic content of Kiss1/Kisspeptin ameliorates the reproductive phenotype despite unfavorable metabolic conditions: The pharmacological "replacement" with kisspeptin was shown to reverse the state of low gonadotropin levels and delayed puberty caused by chronic undernutrition in prepubertal female rats[157]. Thus, the timing of VO was normalized in female rats subjected to peripubertal subnutrition after the chronic icv injection of kisspeptin during such period[157].

Potential neuroendocrine pathways for the metabolic control of puberty

Several lines of evidence suggest the involvement of a potential *leptin-kisspeptin pathway* (**Figure 8**) in the metabolic control of puberty and reproduction: (i) conditions of leptin deficiency reduce the number of kisspeptin immunoreactive cells in the AVPV[166], and the hypothalamic expression of Kiss1 mRNA, especially in the ARC[166,167]; (ii) leptin administration increases the hypothalamic expression of Kiss1 mRNA in animal models of leptin deficiency and in neuronal cell lines[3]; (iii) leptin is able to stimulate ARC Kiss1 neurons through activation of transient receptor potential cation (TRPC) channels in guinea pigs[168]; and (iv) the mRNA encoding the functional LepR is

detected in ARC Kiss1 neurons from mice and ewes[166,167]. The above experimental evidence, as a whole, suggests that leptin can directly modulate hypothalamic Kiss1 neurons. However, it is worth to note that most of the supporting evidence for such function has been obtained in adult animals/models and further studies are required to address the potential interplay between leptin and kisspeptin in the particular context of puberty onset[6]. Interestingly, our initial studies in models of early nutritional manipulation have demonstrated a tight correlation between circulating levels of leptin, hypothalamic expression of the Kiss1 and the timing of puberty[161]. Thus, high levels of leptin have been associated with increased Kiss1/kisspeptin expression and early puberty onset (or vice versa) in female rats[161]; a phenomenon that suggests a relevant role of leptin in the regulation of the hypothalamic Kiss1 system at the time of puberty.

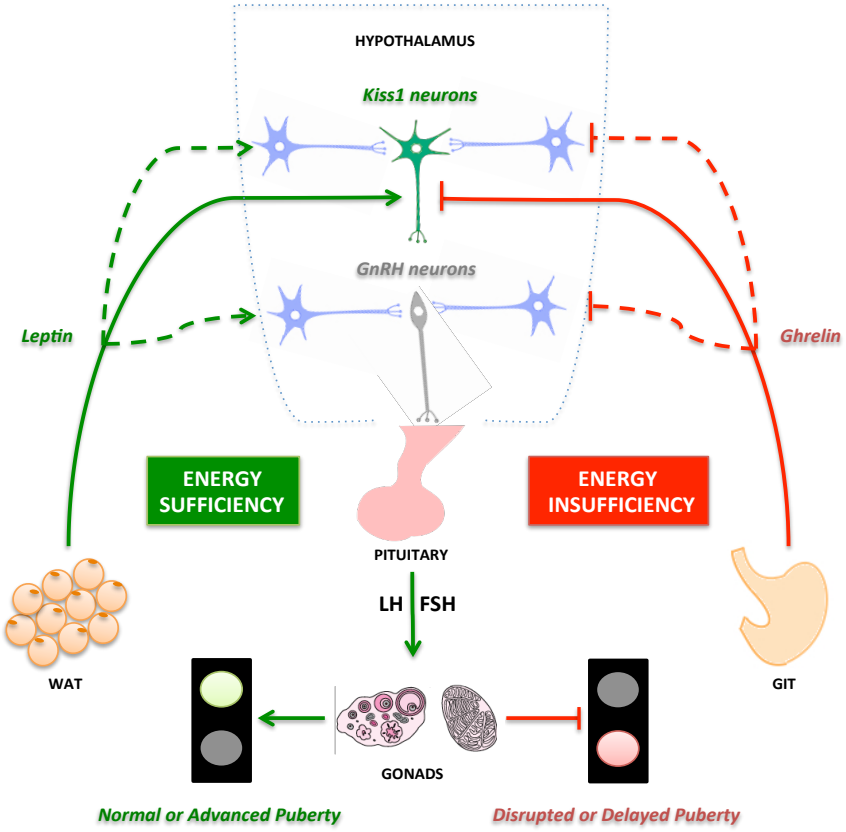


Figure 8: Tentative model for the central integration of the metabolic control of puberty through the hypothalamic Kiss1 neurons. The putative roles of leptin (as prominent signal for energy abundance; permissive/stimulatory factor) and ghrelin (as putative signal of energy insufficiency; inhibitory factor) are highlighted. In addition, the prominent role of Kiss1 neurons as integrators and transmitters of the metabolic information onto GnRH neurons is also represented. (Modified from from *"Metabolic control of female puberty: Potential therapeutic targets", J. M. Castellano et. al. Expert Opinion on Therapeutic Targets, 2016*).

Recent findings, however, support an indirect mode of action of leptin on Kiss1 neurons in the context of pubertal development. For instance, (i) congenital ablation of LepR from Kiss1 neurons does not seem to affect the timing of puberty[169]; and (ii) LepRs are apparently not detected in Kiss1 neurons before puberty[170,171]; yet, a small portion of ARC Kiss1 neurons have been shown to express

functional LepRs in the adulthood[170-172]. Further pieces of evidence that suggest the existence of an indirect connection between leptin and kisspeptin neurons are as follows: (iii) the identification of a novel population of LepR-expressing neurons that do not produce kisspeptin in the ARC and the AVPV[172]; (iv) the recent role documented for the neuropeptide **α**-MSH in mediating the pubertal actions of leptin on Kiss1 neurons in the metabolic control of puberty[173]; and (v) the relevant role suggested for the hypothalamic pre-mammillary nucleus (PMV) in conveying the permissive effects of leptin on female puberty onset[169,174] and, specifically, for the PMV PACAP (Pituitary adenylate cyclase activating polypeptide) neurons, which have been proposed as relevant mediatiors for the reproductive actions of leptin on GnRH release by modulating the activity of kisspeptin neurons[175].

The involvement of a potential ghrelin-kisspeptin pathway (**Figure 8**) in the metabolic control of puberty remains virtually unexplored. Interestingly, emerging evidence suggests that ghrelin may act as putative regulator of Kiss1 expression in female rodents[176,177]. Thus, administration of ghrelin to adult female rats subjected to conditions of normo- and undernutrition was able to significantly reduce the expression of Kiss1 mRNA in the POA; a phenomenon that may contribute to the suppressive actions of ghrelin on the reproductive maturation and function. On the other hand, recent studies involving GHSR-eGFP mice have suggested that the impact of ghrelin on Kiss1 expression at the POA might be indirect, since no expression of GHSR, the functional ghrelin receptor, was observed in the population of kisspeptin neurons located at the AVPV[178]. In contrast, a recent study conducted in OVX female mice demonstrated the expression of GHSR in a subset of Kisspeptin neurons located at the ARC[177]. Indeed, this study also showed that ghrelin is able to acutely depolarize ARC Kiss1 neurons[177]. Overall, the above data suggest that ghrelin could act on hypothalamic Kiss1 neurons to modulate reproductive function; yet, further studies are required to characterize the potential mode of action of ghrelin on Kiss1 neurons, and to what extent such ghrelin-kisspeptin interplay is relevant for the timing of puberty.

Finally, it is worth to mention that recent evidence has suggested a relevant role of GABAergic neurons in mediating the reproductive actions of leptin on female pubertal development[179,180]. Indeed, female mice lacking functional leptin receptors in GABAergic neurons display delayed or absent puberty onset[179,180].

Potential molecular pathways for the metabolic control of puberty

The recent study of the potential molecular mechanisms involved in the metabolic control of puberty has allowed the identification, among others, of two relevant energy sensors that may act as central molecular mediators in such context: the mammalian target for rapamycin (mTOR) and the AMP-activated protein kinase (AMPK).

mTOR has been proposed to be essential in keeping energy balance at the whole body level, among other mechanisms, by transmitting the anorectic effects of leptin[181]. Interestingly, recent

studies from our laboratory have suggested that mTOR also plays a key role in the metabolic control of puberty[159]. Thus, while persistent blockade of central mTOR signaling, by icv administration of rapamycin, delayed the onset of puberty[159], its activation, by chronic icv treatment with L-leucine, stimulated LH secretory responses and partially rescued the state of low gonadotropin levels caused by chronic subnutrition in pubertal female rats[159]. Furthermore, hypothalamic inhibition of mTOR also blocked the permissive/stimulatory actions of leptin on the timing of puberty in leptin-treated female rats subjected to chronic subnutrition[159]. As a whole, these findings strongly support the positive role of mTOR signaling as central molecular transmitter for the actions of leptin in the metabolic control of puberty. Of note, such leptin-mTOR pathway has been shown to modulate the hypothalamic expression of Kiss1 mRNA, as evidenced by the significant inhibition of Kiss1 mRNA levels detected at the ARC and (to a lesser extent) the AVPV of pubertal female rats[159]. However, an indirect mode of action of mTOR in the regulation of Kiss1 has also been suggested by the abscense of phosphor-S6, an essential down-stream element of the mTOR cascade, in Kiss1 neurons[166]. Overall, these observations suggest that a leptin–mTOR–kisspeptin pathway could play a key role in the metabolic regulation of female puberty.

AMPK is a member of the metabolite-sensing protein kinase family with ability to detect changes in the AMP/ATP ratio and hence in the metabolic state of the cell[182,183]. AMPK is activated in conditions of energy insufficiency, i.e., when ATP is consumed, and excess AMP accumulates in the cell. As a result of such activation, different ATP consuming metabolic pathways are inactivated. AMPK signaling has shown to be modulated by leptin and ghrelin, which act as inhibitor and stimulator of AMPK activity, respectively[184]. Interestingly, AMPK and mTOR seem to be mutually regulated. Indeed, AMPK and mTOR have been proposed to reciprocally cooperate in the central control of energy homeostasis[181]. Whether such interaction applies also to the metabolic control of puberty needs to be experimentally proven. Interestingly, emerging evidence indirectly support this possibility. Thus, our preliminary data suggest that activation of central AMPK signaling delays the onset of puberty in female rats; a phenomenon that could be mediated by a partial reduction of Kiss1 mRNA expression in the ARC.

NOVEL MECHANISMS FOR THE CENTRAL CONTROL OF PUBERTY

The complex and sophisticated nature of puberty, and its sensitivity to different regulators, strongly suggest the involvement of diverse but complementary regulatory mechanisms, enabling such a degree of precise control. Recent evidence suggests that epigenetic regulatory mechanisms, such as DNA methylation, histone modifications and small non-coding RNAs (ncRNAs), may be involved in this process[81,185]. Among them, a relevant role for microRNAs (miRNAs) has been recently suggested[13,15].

miRNAs are small (18-25 nucleotides –nt- length), non-coding RNAs that operate as post-transcriptional regulators of the expression of numerous gene targets[186]. In animals, miRNAs act mainly via their capacity to bind to partially complementary sequences in the 3'-UTR of target RNAs, thus resulting in most cases in translation blockade and gene silencing[187]. Eventually, miRNAs may also favor target RNA degradation in animal cells, thus causing also suppressed gene expression. It is worth to mention that some miRNAs may also operate upon recognition sites located in the coding or 5'-UTR regions of certain genes and, despite their predominant inhibitory actions, they can also cause enhanced gene expression.

MiRNAs are encoded by chromosomal DNA and transcribed as long primary transcripts, named pri-miRNAs, which are initially processed into ~70nt stem-loop shaped precursor miRNAs (pre-miRNAs) by the nuclear enzyme Drosha. Subsequently, pre-miRNAs are exported to the cytoplasm and cleaved by the RNAase III enzyme, Dicer, to produce the mature miRNAs, which participate in translational repression or degradation of target mRNAs via interaction with the miRISC complex[188] (**Figure 9**).

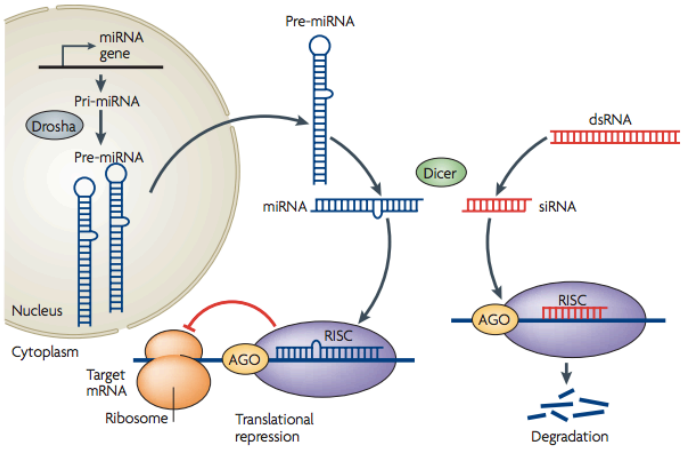


Figure 9: Schematic illustration of miRNA biogenesis. (Taken from *"The chromatoid body: a germ-cell-specific RNA-processing centre", N. Kotaja and P. Sassone-Corsi. In Nature Reviews Mollecular Cell Biology, 2007.)*

It is worth noting that a single miRNA can operate upon recognition sites present in numerous genes, thus enhancing its regulatory capacity. Indeed, according to bioinformatic predictions, one miRNA may have up to 100 different gene targets. In turn, a single gene can be regulated by multiple miRNAs. Additionally, miRNA expression in different tissues is under the control of various regulatory signals, including endogenous factors and environmental cues[188]. Such a multiplicity in terms of miRNAs, potential targets and regulators results in an exponential combination of regulatory mechanisms that endows the system with a considerable degree of plasticity and flexibility, which is optimal for the control of complex biological phenomena, such as the onset of puberty.

Interestingly, different genome-wide association studies (GWAS) unveiled an association of the age at menarche with variability at 6q21, in or near the Lin28B gene[189-192]. Lin28B is a RNA-binding

protein whose major known function is to block the processing of mature miRNAs of the let-7 family[193,194]. Hence, these (indirect) evidences were the first to suggest the potential involvement of miRNAs, of the let-7 family and/or other related groups, in the regulation of puberty. Based on these observations, our group performed qPCR analyses to document the presence of the Lin28/let-7 hub in rat hypothalamus and, if that was the case, its expression profile during the pubertal development. Interestingly, these analyses not only showed a robust expression of Lin28B mRNA, but also its significant decrease from the neonatal to the pubertal period[13]. In addition, the hypothalamic expression of let-7a and let-7b miRNAs was also evaluated in this study; in contrast to Lin28B mRNA, their relative levels significantly increased during postnatal maturation[13]. Importantly, different animal models of perturbed puberty also presented altered ratios of Lin28B/let-7 at the time of puberty. Overall, the above evidence suggested that developmental changes in hypothalamic Lin28/let-7 expression might have a role in the mechanisms leading to puberty onset.

Interestingly, a more recent study has documented a miRNA–mediated mechanism responsible for the switch from repression to induction of GnRH expression that leads to the onset of puberty[15]. This switch takes place during the infantile period and corresponds to a centrally-driven gonad-independent activation of the reproductive axis known as "mini-puberty"[15]. Two miRNAs species are specifically involved in this process: miR-200/429 family, whose members are significantly increased during the infantile period and enhanced in GnRH neurons, and miR-155, which is expressed in other hypothalamic neurons and seems to mediate the secretion of nitric oxide that occurs concomitantly upstream of GnRH neurons. The target genes directly or indirectly regulated by miR-155 and miR-200/429 include Cebpb, Zeb1, Pou2f1 and Meis1. The activation of Cebpb, Zeb1 or the inhibition of Pou2f1 and Meis1 (specific targets for Zeb1) is required for the increase of GnRH expression that occurs from mini-puberty to the onset of puberty. Interestingly, blocking the binding of the hypothalamic miR-200/429 to the 3′UTR region of Zeb1 mRNA during the infantile period suppresses the transcriptional activity of GnRH promoter and leads to alterations in the onset of puberty. As additional proof of the potential relevance of miRNAs in the central control of puberty, it is worth to note that transgenic mice lacking the protein Dicer, which is responsable for processing precursor miRNAs into their active smaller forms, in GnRH neurons, lack the hypothalamic mRNA expression of GnRH and are infertile[15].

4. NOVEL SIGNALS FOR THE CENTRAL CONTROL OF PUBERTY

CERAMIDES

Ceramides are a family of lipids that consist of a sphingoid long-chain base covalently linked to a fatty acid. Ceramides are precursors for the predominant sphingolipids in the cell, including sphingomyelin and gangliosides. This sphingolipid family includes over 4000 distinct species (www.lipidmaps.org) that are integral components of cell membranes.

Ceramides differ from each other by length, hydroxylation, and saturation of both the sphingoid base and fatty acid moieties. Sphingoid bases are of three general chemical types: (i) *sphingosine*, which has a trans-double bond at the C4-5 position; (ii) *dihidrosphingosine* or *sphinganine*, which presents a saturated sphingoid backbone and are considered as the inactive precursors of ceramide; and (iii) *phytosphingosine*, which has a hydroxyl group at the C4 position[195].

The fatty acid chain of ceramides varies widely in composition. The acyl chain lengths range from 14 to 26 carbon atoms; yet, the most common fatty acids are palmitic (C16:0) and stearic (C18:0) non-hydroxy fatty acids. The fatty acids are commonly saturated or mono-unsaturated [196].

Pathways for ceramides generation

Ceramides can be synthesized by three major routes: via the *de novo* synthesis, via hydrolysis of complex sphingolipids and via salvage pathway[197] (**Figure 10**). While the activation of different catabolic enzymes yields ceramides within a few minutes, the *de novo* synthesis produces ceramide in several hours[198]. Different extra- and intracellular stimuli determine the pathway used for ceramide generation resulting in distinct subcellular localization of ceramides and different biochemical and cellular responses.

The ***de novo synthesis*** of ceramide begins in the endoplasmic reticulum with the condensation of palmitoyl-CoA and serine, catalyzed by the multimeric enzyme *serine palmitoyltransferase (SPT)*, to produce 3-ketosphinganine[199]. The 3-ketosphinganine produced by the SPT reaction is a short-lived intermediate that is rapidly converted to sphinganine by *3-ketosphinganine reductase* (3KSN). The subsequent N-acylation of sphinganine by a family of *ceramide synthases* (CERS1–6) produces dihydroceramides, and much of the diversity in the sphingolipid pool results from this reaction. The six different mammalian CERS enzymes produce dihydroceramides of variable acyl-chain lengths, ranging from 14 to 34 carbon atoms[200]. These enzymes differ in substrate specificity and tissue distribution, allowing the formation of distinct sphingolipid pools in different tissues and cell types. The selectivity of different CERS isoforms to synthesize different ceramide species is important since ceramide with specific acyl chain lengths might mediate different responses within cells[201]. Finally, *dihydroceramide desaturases* (DES1 and 2) insert a double-bond that imparts many of the unique biophysical properties of the sphingolipid[10]. DES1 is the dominant enzyme in most tissues, with DES2 making phytosphingolipids in the skin and gut.

The biophysical properties of ceramides, which include high hydrophobicity and low polarity, limit its free circulation inside the cell or in solution. However, the cell needs to transport these new ceramides formed by the *de novo* synthesis from the luminal face of the ER to the Golgi compartment to generate complex sphingolipids[202]. To this end, the cell employs two major mechanisms: via the protein ceramide transfer protein (CERT) or via vesicular transport.

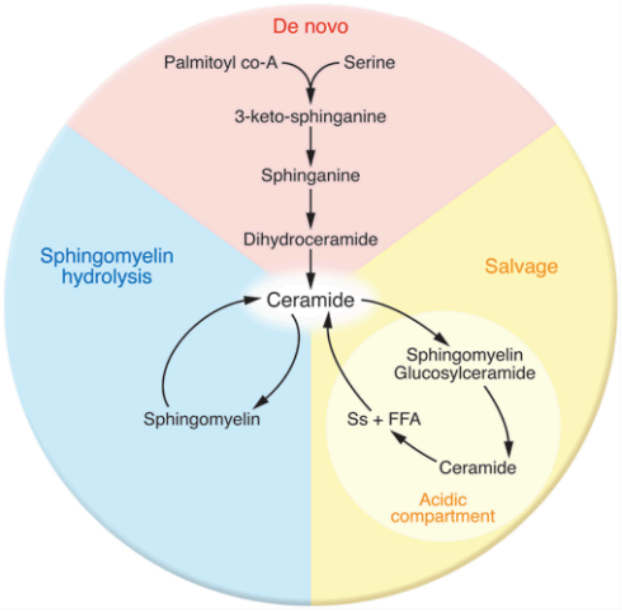


Figure 10: Representative scheme of the three major pathways for ceramide synthesis. (Taken from *"Ceramides as modulators of cellular and whole-body metabolism", B.T. Bikman and S.A. Summers. Journal of Clinical Investigation, 2011).*

The CERT is a cytosolic protein composed by different functional domains that determine its function. The N-terminus of CERT contains a PH domain, which is able to recognize PI4P on acceptor Golgi membranes and therefore allows for directed transport to the Golgi. In the middle of the protein a FFAT domain serves for donor membrane recognition. The C-terminus of CERT contains a START domain, which provides a hydrophobic pocket responsible for the direct binding of ceramide and allows for its delivery to the Golgi through an aqueous environment. CERT mediates the transfer of ceramides containing C14-C20 acyl chain sphingomyelins in many tissues and cell lines. CERT works as mediator of sphingolipids homeostasis.

The alternative pathway for transfering ceramides to the Golgi is based on vesicular transport. However, our knowledge about the regulation of this pathway, which is considered the major mechanism for delivering ceramides to the Golgi for glycosphingolipid synthesis, is still incomplete.

Ceramides committed to the formation of sphingomyelins can reach the Golgi carried by the CERT in a non-vesicular manner[202], whereas ceramides destined for conversion to glycosphingolipids and sphingomyelins appear to reach the Golgi only via the vesicular route[202]. The addition of sugar groups to ceramides is catalyzed by membrane bound glycosyltransferases and it is restricted to the ER-Golgi complex[203]. The synthesis of most glycosphingolipids begins with glycosylation of ceramide to form glucosylceramide at the cytosolic surface of the Golgi[204].

Once delivered to the Golgi apparatus, ceramides spontaneously translocate from the cytosolic to the luminal leaflet for sphingomyelin synthesis. Formation of sphingomyelins from ceramides is catalyzed by sphingomyelin synthase (SMS)[205] that transfers the phosphocholine headgroup from phosphatidylcholine onto ceramides yielding sphingomyelin as a final product and diacylglycerol (DAG) as a side product[206]. Two different isoforms of SMS have been described: SMS1, which is located in the lumen of the cis/medial Golgi[207] and is considered the key enzyme for the *de novo* synthesis of sphingomyelin[208]; and SMS2, which resides in the plasma membrane[206] and could play a more specific role in signal transduction events.

As we previously mentioned, ceramides can also be produced by ***hydrolysis of complex sphingolipids***. Thus, ceramides derived from sphingomyelin catabolism require the activation of sphingomyelinases (SMases)[209], which hydrolyze the phosphodiester bond of sphingomyelin yielding soluble phosphorylcholine and ceramide[210]. Based on their optimum pH, subcellular distribution and regulation, we can distinguish the following SMases: (i) the acid sphingomyelinases, which are lipases localized in lysosomes that are required for the turnover of cellular membranes[211]; (ii) the neutral sphingomyelinases, which are membrane bound enzymes that include several isoforms, such as neutral sphingomyelinase 1, localized in the membranes of the ER and highly enriched in kidney[212], neutral sphingomyelinase 2, located in the plasma membrane and specifically highly expressed in brain[213, 214], and neutral sphingomyelinase 3, ubiquitously present in all cell types and distributed mainly in the ER and Golgi membrane[215]; and (iii) the alkaline sphingomyelinases (aSMase), which exist only in intestinal cells and are activated by bile salts[216]. Although the function of these multiple isoforms is still elusive, their membrane localization has led to speculation that they may contribute to the modification of local microdomains in the membrane organization during vesicle formation, transport, and fusion[217].

Finally, ceramides can be also generated by an alternative acyl-CoA-dependent route, which is called the "***salvage pathway***". This sphingolipid recycling pathway requires the reverse activity of the enzyme ceramidase, which catalyzes the hydrolysis of ceramides to generate free sphingosines and fatty acids. It is important to note that whereas sphinganine is generated by *de novo* sphingolipid biosynthesis, free sphingosine seems to be derived only via turnover of complex sphingolipids, more specifically by hydrolysis of ceramides[199]. Free sphingosine is probably trapped at the ER-associated membranes where it undergoes re-acylation (condensation with a fatty-acylCoA) to again generate ceramides. This "reverse" activity is carried out by the same ceramidase[218]. Based on their optimum pH and subcellular distribution, we can distinguish the following ceramidases: (i) the acid ceramidases, which are lysosomal[213]; (ii) the neutral/alkaline ceramidases, which have been detected in mitochondria and nuclear membranes[219,220]; and (iii) the pure alkaline ceramidases, which have been localized in the Golgi apparatus and ER[221]. Furthermore, ceramidases has been detected in rat brain[222], mouse liver and human kidney. Overall, the above variability, in terms of subcellular localization and tissue distribution, suggests that ceramidases may have diverse functions in the biology of the cell.

Roles of ceramides

Today, it is known that ceramides are not only structural components of cell membranes but also active molecules that exert a wide range of biological functions in many of the most critical cellular events, including growth, differentiation, apoptosis and oncogenesis[223]. Here, we will outline some of the most relevant functions of ceramides:

Lipid raft and cell signalling. Ceramides may act at the level of lipid rafts in transducing external signals. Rafts are the primary sites of action of the enzyme sphingomyelinase for releasing ceramides from sphingomyelins[224]. The nature of ceramide has a tremendous influence on membrane structure. Indeed, long-chain saturated ceramide molecules form liquid ordered domains that induce lateral phase separation of fluid phospholipid bilayers into regions of liquid-crystalline (fluid) phases. Furthermore, the small size of ceramide polar headgroup results in a low hydration and allows ceramide molecules to pack tightly avoiding any interference with surrounding lipids[225].

The ceramide-enriched membrane platforms serve as clustering components to achieve a critical density of receptors involved in signaling. This high density of receptors seems to be required for effective transmission of the signal into cells. The neuronal plasma membrane is particularly enriched in lipid rafts[226]. Lipid and protein raft composition differs according to neuronal developmental stage. Thus, mature neuron lipid raft content is higher than that of immature neurons and astrocytes. Synaptic proteins such as synaptophysin or synaptotagmin localize in lipid rafts[227] and lipid rafts are critical for maintaining the stability of synapses and dendritic spines[228]. Neurotransmitter signaling seems to occur through a clustering of receptors and receptor-activated signaling molecules within lipid rafts. Aberrant organization of sphingomyelin and cholesterol in rafts has been linked to the loss of synapses and changes in nerve conduction[228]. Depletion of sphingolipids or cholesterol leads to gradual loss of inhibitory and excitatory synapses and dendritic spines[228]. Rafts also play an important role in neuronal cell adhesion, localization of neuronal ion channels, and axon guidance.

Apoptosis. The role of ceramides in apoptosis is extensive, complex and unclear. While an increase of ceramide levels leads to cell death, its depletion can reduce the progression of apoptosis[223]. Therefore, ceramides may present a dual role within the cell: protection and cell sustenance at low concentrations but death and threat when over-produced. This fact outlines the importance for cells to maintain a strict ceramide balance, which is indispensable for proper function, among others, of the central nervous system (CNS)[223].

Ceramides can induce apoptosis via different routes. The hydrolysis of sphingomyelins is known to be a very important pathway for production of pro-apoptotic ceramides[229]. In the same vein, the *de novo* synthesis pathway has also been reported to be relevant in the generation of a signaling pool of ceramides leading to cellular apoptosis[230, 231]. While sphingomyelin hydrolysis generates a rapid and transient increase of ceramides and results in formation of ceramide-enriched membrane platforms, the ceramide *de novo* pathway requires multiple enzymatic steps and it is responsible for a slow but robust accumulation of ceramides over a period of several hours. These two pathways can induce apoptosis independently or jointly.

Aging. Several studies support the role of ceramides in inducing senescence and in activating genetic/biochemical pathways involved in aging. Accumulation of ceramides occurs normally during

development and aging in single cells[232]. Indeed, young cells treated with exogenous ceramide exhibit a senescent-like phenotype[233]. Furthermore, a significant change in ceramide metabolic enzyme activities seems to occur in specific organs or even in specific cell types with aging[232,234]. The activities of the sphingolipid catabolic enzymes (sphingomyelinase and ceramidase) seem to change more robustly than that of the anabolic enzymes (sphingomyelin synthase and ceramide synthase).

Metabolic disorders. For some time, ceramide production was thought to be controlled by the availability of its two initial substrates, palmitate and serine. Thus, the oversupply of these nutrients might contribute to the upregulation of ceramides in obesity. However, recent studies have revealed that tissue ceramide levels may be also regulated by hormonal cues. Interestingly, while inflammatory agents are upregulated in obesity and are important drivers of ceramide production, the adipose hormone adiponectin exerts its broad spectrum of actions by degrading ceramide via ceramidase activation.

Inflammation and ceramide synthesis. Obesity is associated with chronic low-level inflammation. Saturated fatty acids activate or enhance signaling through receptors involved in innate immunity (e.g., Toll-like receptors. TLRs), thus upregulating cytokine synthesis and secretion[235]. Furthermore, macrophages infiltrate the expanded adipose depot, which then expresses different cytokines and other macrophage markers[236]. This inflammatory environment is crucial for the upregulation of ceramides. Indeed, the presence of TLR4 signaling network in a subset of tissues has shown to be a prerequisite for palmitate-mediated induction of ceramides[237,238]. The mechanism by which ceramides might contribute to inflammation-induced insulin resistance and diabetes is through the activation of the nucleotide-binding domain, leucine-rich-containing family, pyrin domain containing 3 (NLRP3) inflammasome[239,240].

Adiponectin and ceramide degradation. Adiponectin is a member of the family of adipose tissue-related hormones, known as adipokines, which elicits a broad spectrum of protective actions (e.g. antidiabetic, antiatherogenic, antiinflammatory, and antiangiogenic)[241]. Notably, its transgenic overexpression in obese, leptin-deficient mice induces a metabolic alteration whereby animals accumulate enormous amounts of adipose tissue but are protected from insulin resistance and metabolic disorders[242]. Interestingly, it has been found that adiponectin increases cellular ceramidase activity, and the anti-apoptotic actions of this adipokine could be blocked by adding ceramidase inhibitors or by deleting a crucial residue in the predicted ceramidase motif[243]. Furthermore, a recent study has proposed the existence of a Fibroblast Growth Factor 21 (FGF21)–Adiponectin–Ceramide Axis that drives hypermetabolic responses to increase glucose and lipid utilization[243]. Indeed, FGF21 was found to selectively lower ceramide levels while simultaneously inducing expression of adiponectin[244].

In addition to the above, it is worth to emphasize that ceramides also show a key role in relevant processes for the generation of obesity and its metabolic disorders, such as endoplasmic reticulum stress (ER stress) and insulin resistance.

ER stress. The term ER stress refers to the alterations of the protein-folding functionality of the ER, which activates a complex signaling network named the unfolded protein response (UPR), leading to the attenuation of protein synthesis, upregulation of ER-folding machinery (e.g. chaperones), and degradation of irreversibly misfolded proteins. Interestingly, a very recent study suggests that this phenomenon, which is often linked to obesity, is mediated by central ceramide signaling[10]. In particular, this study demonstrated that ceramides are able to induce hypothalamic lipotoxicity and ER stress, leading to sympathetic inhibition, reduced brown adipose tissue (BAT) thermogenesis and feeding-independent weight gain[10]. Furthermore, the same study showed that genetic modulation of the ceramide-induced ER stress pathway in a specific hypothalamic nucleus, the VMH, modulates energy balance by influencing BAT thermogenesis, in a sympathetic nervous system (SNS)-mediated manner, and insulin sensitivity, as well as by promoting an overall improvement of the metabolic phenotype of leptin and insulin resistant obese Zucker rats[10].

Insulin resistance. A role for ceramides in insulin resistance was disclosed by observations that ceramides inhibited insulin-stimulated glucose transport. This action resulted from the ability of ceramides to repress insulin-mediated stimulation AKT/protein kinase B (PKB), a serine/threonine kinase that is an obligate intermediate in anabolic signaling[245,246].

Using cultured cell models of insulin resistance, different studies demonstrated that blocking ceramide synthesis by pharmacological ablation and/or knockdown of the genes encoding SPT, CERS, or DES1 can restore insulin signaling to AKT/PKB in cultured cells bathed in excess concentrations of palmitate[247,248]. In the same vein, overexpressing acid ceramidase to catalyze ceramide deacylation had similar effects[248].

In rodents, pharmacological inhibition of SPT, CERS, and/or DES1 prevented insulin resistance caused by lard infusion, dexamethasone, high fat feeding, and leptin or leptin receptor deficiency[9]. Genetic ablation of one or more alleles of SPTLC2, CERS6, or DES1 was also found to reduce/ablate insulin resistance in murine models[9].

Interplay of hypothalamic ceramides with leptin and ghrelin

Recent evidence documents the potential role of hypothalamic ceramides as relevant mediators for the anorexigenic and orexigenic actions of leptin and ghrelin, respectively, in the control of food intake and energy balance.

Leptin treatment has been shown to induce a significant decrease in the ARC ceramide levels of adult male rats. This decrease is crucial for leptin-mediated anorectic effects and involves an increase

in the levels of malonyl-CoA, which is an intermediate in fatty acid *de novo* biosynthesis, and the subsequent inhibition of the enzyme carnitine palmitoyltransferase-1C (CPT1C), which is key for the mitochondrial fatty acid ß-oxidation[11]. This perturbation in malonyl-coA/CPT1C signaling reduce the hypothalamic *de novo* synthesis of ceramides, resulting in the downregulation of the orexigenic neuropeptide Y (NPY) and its transcriptional regulator Bsx, and, ultimately, the inhibition of food intake[11].

Central ghrelin administration, unlike leptin, elicits a significant upregulation of ceramide levels in the medial basal hypothalamus via CPT1C. This increase is required to induce hyperphagia and the hypothalamic expression of the orexigenic neuropeptides, agouti-related protein (AgRP) and NPY. Indeed, central inhibition of ceramide synthesis with myriocin was able to block the orexigenic actions of ghrelin and normalized the levels of AgRP and NPY, as well as their key transcription factors phosphorylated cAMP-response element–binding protein (pCREB) and forkhead box O1 (FoxO1). Furthermore, central injection of ceramide increased the levels of AgRP and NPY and induced food intake in CPT1C KO mice, which had the ghrelin canonical pathway constitutively activated[12].

Because the above findings suggest the importance of ceramide signaling in mediating the metabolic actions of leptin and ghrelin, it is reasonable to think that hypothalamic ceramides may act also as putative mediators of additional relevant actions of those hormones in other biological contexts, such as the central control of puberty onset. However, the study of this intriguing possibility, that might have an enormous impact in translational research due to the increase prevalence of pubertal alterations related to metabolic disorders, has not been addressed so far.

miR-30b/MAKORIN 3 PATHWAY

MKRN3 is an intronless gene located on human chromosome 15q11–13 (chromosome 7C in mouse). This gene is maternally imprinted, that is, the copy of the gene derived from the mother is not expressed; a phenomenon that is associated with 5′CpG island methylation[249-251]. The MKRN3 gene was first cloned in 1999 during a study of the Prader–Willi/Angelman syndrome (PW/AS) critical region[251]. This work identified a cDNA in the PW/AS region encoding a zinc finger protein, initially named zinc finger protein 127 (ZNF127) and later renamed MKRN3. Interestingly, an antisense transcript was concomitantly identified and named ZNF127AS (MKRN3-AS1)[251]. This antisense transcript is not translated and, like other untranslated antisense transcripts, has been speculated that may regulate the expression of the sense transcript (MKRN3).

MKRN3 encodes a ribonucleoprotein consisting of a central RING finger motif (C3HC4), two amino-terminal C3H zinc finger motifs followed by the unique pattern of conserved Cys–His residues called a Makorin zinc finger motif, and a carboxy-terminal C3H zinc finger motif[251] (**Figure 11**). MKRN3 is highly conserved among species, and the mouse and human MKRN3 amino acid sequences share 69% identity, which defines the percentage of amino acids that match directly in the

alignment, and 82% similarity, which considers the sum of both identical and similar matches (residues that have undergone conservative substitution and, therefore, do not compromise the translated protein) between the aligned sequences[251].

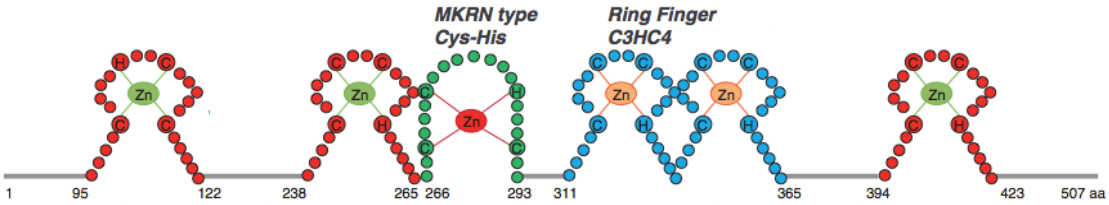


Figure 11: MKRN3 protein structure. Zn: zinc; H: histidine; C: cysteine. The C3H zinc finger motifs are shown in red, the MKRN-specific Cys–His domain is shown in green and the C3HC4 RING finger motif is shown in blue. (Modified from *"A new pathway in the control of the initiation of puberty: the MKRN3 gene, A.P. Abreu et al. In Journal of Molecular Endocrinology, 2015).*

MKRN3 presumptively possesses ubiquitin-protein isopeptide ligase (E3) activity, conferred by the presence of the highly conserved C3HC4 RING finger domain. The tandem repeat of C3H zinc fingers may provide high-specificity RNA binding, and the unique Cys–His makorin motif has been suggested to be a DNA-binding domain. This category of enzymes (ubiquitin-protein isopeptide ligase E3) mediates the transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to target protein substrates[252]. As a result of this process, known as ubiquitination, the target protein can be subjected to different actions, varying from proteasome-dependent proteolysis to modulation of protein function and/or localization[252].

MKRN3 is ubiquitously expressed in adult human tissues, with the highest expression levels in the testis. In the fetus, it is highly expressed in the CNS and in post-meiotic sperm germ cells, particularly in round spermatids[251].

Potential role of MKRN3 in the central control of puberty

Evidence from genetic studies. The potential role of MKRN3 in the onset of puberty was first suggested in 2013 after a comprehensive genetic study of a cohort of 40 members of 15 families with central precocious puberty (CPP)[16]. The whole-exome sequencing analysis of these patients resulted in the detection of four deleterious MKRN3 mutations, three frame-shift mutations and a missense mutation, in five of these families (33%). Furthermore, both sexes were equally affected by MKRN3 mutations (eight girls and seven boys).

More recently, Macedo et al. studied 215 unrelated children with CPP from three different University Medical Centers and identified five novel heterozygous mutations in 8 unrelated Brazilian girls, including four frameshift variants and one missense mutations[16]. In all cases, it was demonstrated that the mutant allele was paternally inherited in all families with MKRN3 mutations. In the same vein, other investigators have also reported MKRN3 defects associated with familial CPP.

Thus, Settas et al. described a novel heterozygous missense mutation (p.Cys340Gly) in MKRN3 in two Greek siblings, a girl with CPP and a boy with early puberty[17]. Similarly, Schreiner et al. identified two heterozygous MKRN3 mutations (p.Glu111* and p.Ala162Glyfs*14) in patients of two German families[18]. Finally, de Vries et al. found a novel missense mutation (p.His420Gln) in four siblings[253].

Currently, 12 distinct loss-of-function mutations of MKRN3 have been described in 30 patients (22 girls and eight boys) with CPP from 17 families of different ethnicities. Notably, 8 of these mutations encode either premature stop codons or frameshift mutations. The four missense mutations (p.Cys340Gly, p.Arg365Ser, p.Phe417Ile, and p.His420Gln) were located within a zinc finger motif or a RING finger motif, regions predicted to be involved in RNA binding and ubiquitin ligase activity of the protein, respectively, and essential for protein function. Interestingly, most of the MKRN3 mutations (64%) were located in the amino-terminal region of the protein, which is encoded by a poly-C-rich sequence, suggesting that this area may be a potential hotspot.

The potential relevance of MKRN3 in the timing of human puberty has been reinforced recently by a large genome-wide and custom-genotyping arrays conducted in up to 182.416 women of European descent from 57 studies[254]. These analyses found robust evidence for 123 signals at 106 genomic loci associated with age at menarche. Three of these loci were located in imprinted regions, including the MKRN3 locus, demonstrating parent-of-origin-specific associations concordant with known parental expression patterns[254]. This study suggests that not only are rare variants in MKRN3 associated with CPP, but also more common variants/polymorphisms may be associated with changes in the timing of puberty (as reflected by the age of menarche) within the normal range and within the general population.

Evidence from expression analyses. Interestingly, recent studies conducted in mice have demonstrated that the hypothalamic expression of Mkrn3 mRNA and protein is significantly reduced before puberty initiation[16,26]. This decline in Mkrn3 expression indirectly suggests that this peptide may repress the initiation of puberty through the inhibition of GnRH secretion during the prepubertal quiescent period.

Evidence from serum determinations. Recent studies have demonstrated that serum levels of MKRN3 decline before the pubertal onset in healthy girls and boys[19,23-25]. However, while the change in MKRN3 levels appears biphasic in boys, with a rapid decline prior to the onset of puberty and slower changes when puberty progresses, the decline in serum MKRN3 continued to mid-puberty in girls[23]. These findings are in good agreement with evidence coming from expression studies and support the potential repressive role of MKRN3 in the control of puberty of both sexes.

Potential role of miR-30b in the regulation of MKRN3 function

Although the above experimental evidence strongly suggests the potential involvement of MKRN3 as a repressor of puberty onset, further studies are required to elucidate the central

mechanisms whereby this protein may modulate the timing of puberty and its potential roles on pubertal alterations linked to extreme endogenous and environmental conditions (e.g. sex steroid/metabolic status). Importantly, the fact that the 3'-UTR region of MRKN3 has 90% of identity between mice and humans suggests not only the functional significance of this region for MKRN3 biological actions but also the potential role for miRNAs with ability to bind to this 3'-UTR region in the regulation of MRKN3 activity[255]. This molecular feature of MKRN3 gene, along with the recent role proposed for different miRNA regulatory systems in the central control of puberty, encourages the search for potential MKRN3-regulatory miRNAs and the study of their potential function in the central control of puberty. In this context, it is worth to note that our initial bioinformatic analyses of the 3'UTR of MKRN3 (*see "Results"* section) have identified three predictive binding sites, broadly conserved among vertebrates, for the microRNA, miR-30b. However, the potential functional link between MKRN3 and miR-30b in the regulation of relevant biological functions, in general, and the central control of puberty, in particular, remained completely unknown and has been the subject of specific analyses in this Thesis.

OBJECTIVES

OBJECTIVES

Puberty is a key developmental period when sexual and somatic maturation is achieved, and reproductive capacity attained. Today, it is assumed that the complex and sophisticated nature of puberty requires the involvement of precise regulatory mechanisms highly sensitive to different endogenous factors and environmental cues. However, our current knowledge about those mechanisms and their roles in the timing of puberty and its potential alterations remains incomplete, as most of such mechanisms are not yet fully characterized. The importance of understanding these mechanisms is emphasized by the trends for an earlier initiation of puberty linked to the escalating prevalence of childhood obesity documented by different epidemiological studies conducted in Europe and USA. This phenomenon has become a major health problem, which is coupled to different adverse outcomes and diseases later on life.

In the above context, the **GLOBAL AIM** of this Doctoral Thesis is to characterize novel regulatory mechanisms involved in the physiological control of puberty, such as central ceramide signaling and the miR-30b/Mkrn3 pathway, and to elucidate their potential involvement in the alterations of pubertal development frequently linked to unfavourable (mainly metabolic) conditions. This global aim is broken down into the following **SPECIFIC OBJECTIVES**:

- To evaluate the potential contribution of central ceramide signaling to the physiological control of puberty and its involvement in the pathophysiology of precocious puberty linked to early overnutrition.
- To dissect out the mechanism(s) whereby central ceramide signaling may influence the control of puberty, including its potential interplay with leptin and kisspeptin.
- To explore the potential role of the miR-30b/Mkrn3 pathway in the central control of puberty and its contribution to alterations in the timing of puberty linked to adverse endogenous and/or environmental conditions.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. ANIMALS

Male and female Wistar rats bred in the vivarium of the University of Cordoba were used in this Thesis. The day the animals were born was considered day 1 of age. The animals were kept under constant conditions of light (14h of light, from 7:00 am) and temperature (22°C), unless otherwise stated. The animals were weaned on postnatal day (PND) 21 and were provided with free access to tap water and pelleted food (A04, Panlab), unless otherwise indicated.

All experiments and animal protocols included in this Thesis were reviewed and approved by the Ethics Committee of the University of Cordoba and were conducted in accordance with European Union normative for the use and care of experimental animals (Directive 2013/53/UE on February 2013).

2. DRUGS

Myriocin (MYR; specific inhibitor of serine palmitoyltransferase enzyme; Ref: M1177), N-hexanoyl-D-sphingosine or C6 ceramide (CER C6; cell-penetrating ceramide precursor; Ref: H6524), GnRH (Ref: L7134), and ß-estradiol 3-benzoate (EB; Ref: E8515) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Kisspeptin (110–119)-NH_2 (termed kisspeptin-10 or KP; Ref: 048-65) was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA). Recombinant rat leptin (LEP; CYT-227) was obtained from ProSpec-Tany TechnoGene Ltd. (Ness Ziona, Israel).

For in vivo experiments: (i) GnRH, KP-10 and LEP were disolved in physiological saline (sodium chloride 0.9%); (ii) MYR and CER C6 were disolved in 70% dimethyl sulfoxide (DMSO; Ref 1096780100); and (iii) EB was disolved in olive oil. For in vitro experiments: GnRH, KP-10, MYR and CER C6 were dissolved in Dulbecco Modified Eagle medium (DMEM; Ref: 31053-028). The doses of the different drugs used in intracerebroventricular (icv) injection (MYR, CER C6, KP or LEP) were administered in a final volume of 5µl.

3. GENERAL EXPERIMENTAL PROCEDURES

SAMPLE COLLECTION

For analyses involving real time polymerase chain reaction (PCR), western-blot (WB) and high-performance liquid chromatography (HPLC), hypothalami were dissected out, immediately upon the decapitation of the animals, by a horizontal cut ~ 2mm in depth with the following limits: 1mm anteriorly from the optic chiasm, the posterior border of mammillary bodies, and the hypothalamic fissures. In addition, selected expression analyses were conducted using either the preoptic area

(POA) or the medial basal hypothalamus (MBH). Dissection of these hypothalamic regions was conducted as previously recommended[256]: (i) the POA was dissected by a transverse cut behind the optic chiasm (OC) and two oblique cuts initiated on each lateral edge of the OC and intersecting at a point anterior to the decussation of the optic nerves; and (ii) the MBH was dissected by two lateral cuts along the hypothalamic sulci, one posterior cut along the rostral border of the mammillary bodies and one anterior cut immediately behind the OC. The tissues were frozen in liquid nitrogen and stored at -80°C until used for PCR, WB or HPLC analyses.

For hormone assays, blood samples were obtained by either jugular venipuncture or decapitation of the animals. Serum samples were separated by centrifugation at 3000 g for 20 min and stored frozen at -20°C until used for hormone determinations.

PHENOTYPIC EVALUATION OF PUBERTAL MATURATION

Somatic and reproductive indices of female pubertal development were evaluated as previously described[257], including (i) body weight (BW); (ii) food intake; (iii) age of vaginal opening (VO), a consensus external marker of puberty in rodents; (iv) age of first estrus, a marker of first ovulation in rodents; (v) uterine and ovarian weights; and (vi) serum LH and FSH levels. VO was monitored daily from the beginning to the end of the experiments. At the latter time, uterine and ovarian weights were recorded, and serum hormone levels were assayed. Once vaginal opening occurred, vaginal lavages were performed daily to identify the occurrence of the first estrus, which in rodents is manifested by a predominance of cornified cells. Further assessment of the age of puberty was achieved by histological analysis of the ovary, as described in detail below.

OVARIAN HISTOLOGY

For ovarian histological analysis, ovarian samples (including the oviduct and the tip of the uterine horn) were fixed for at least 24-h in Bouin solution, and subjected thereafter to dehydration and embedded in paraffin wax. Serial (7 µm-thick) sections were cut, stained with hematoxylin and eosin, and evaluated under the microscope, using previously validated procedures in our group[258]. Precise assessment of pubertal progression was conducted using a scoring method (Pub-Score), recently validated by our team[259], based on histometric analyses of follicular and corporal lutea development (**Figure 12**). According to this method, precise dating of pubertal maturation is based on the combined analysis of follicular development and corpus luteum dynamics (the later, for animals that has completed first ovulation). To this end, in non-ovulating animals, the most advanced healthy antral follicle class, from small follicles measuring less than 275 µm in diameter to antral follicles (from F1 to F5) was determined, allowing to date pre-pubertal maturation from stage -5 to -1 (representing the time expected until the first ovulation). In addition, for animals that had undergone ovulation, dating of corpora lutea (CL), as morphological sign of ovulation, was also implemented, based on major histological features, allowing staging of pubertal timing at one-day intervals, from

+1 (equivalent to CL1) to +5 (equivalent to CL5). Note that negative scores denote expected days until first ovulation, whereas positive scores indicate days after the first ovulation, therefore providing an integral assessment of the stage of pubertal maturation, even in animals that have not completed puberty.

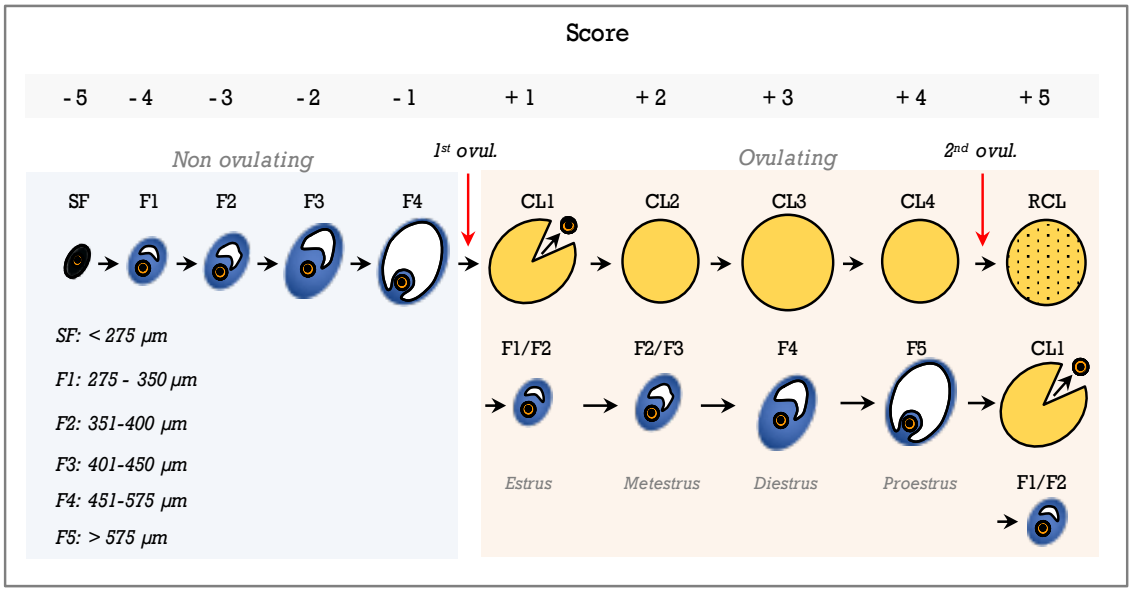


Figure 12: Schematic drawing of ovarian maturation stages, from non-ovulating to ovulating, and their corresponding Pub-scores. (Modified from *"Development and validation of a method for precise dating of female puberty in laboratory rodents: The puberty ovarian maturation score (Pub-Score), F. Gaytán et al., Scientific Reports 2017).*

4. GENERAL SURGICAL PROCEDURES

CANNULATION AND INTRACEREBROVENTRICULAR ADMINISTRATION

For intracerebral injections, standard procedures of cannulation of the lateral cerebral ventricle, followed by intracerebroventricular (icv) administration of different compounds, were implemented in pre- and peripubertal female rats[260]. Animals were cannulated 24 hours before the beginning of the pharmacological studies. To this end, cannulas (INTRADEMIC polyethilene Tubing, Becton Dickinson, Sparks, MD, USA) were inserted to a depth of 2 mm beneath the surface of the skull, with an insert point at 1 mm posterior and 1.2 mm lateral to Bregma according to a rat brain atlas[261]. After cannulation, the animals were housed in individual cages until the end of the experiments. Daily inspection of the cannulae was conducted in each animal in order to exclude those showing obvious displacement or de-attachment. When animals were injected at the late infantile period (PND15), the compounds were directly administered into the lateral cerebral ventricle following the above coordinates. No cannulae were implanted in this particular case.

5.GENERAL ANALYTICAL PROCEDURES

REVERSE TRANSCRIPTION (RT) AND REAL-TIME PCR (mRNA and miRNA)

Total RNA extraction*:* Total RNA was extracted from the ovary and hypothalamus using TRIsure isolation reagent (Bioline Reagents Ltd., UK) according to manufacturer's instructions. RNA concentration of each sample was determined using a Nanodrop ND-1000 v3.5.2 spectrophotometer (Nanodrop Technology®, Cambridge, UK). RNA quality was determined by visualizing the 28S/18S ribosomal RNA ratio (2:1) in an agarose gel (1%) elecrophoresis.

Reverse transcription for mRNA*:* In order to remove potential genomic DNA contamination from intronless target genes, 1 µg of total RNA was DNAse-treated following manufacturer's recommendations (172-5034; iScriptTM gDNA Clear cDNA Synthesis Kit; Bio-Rad Laboratories Inc., USA). Each DNA digestion consisted of 1 µg total RNA and 2 µl DNAse master mix (iScript DNAse + DNAse Buffer) in a final volume of 16 µl. This reaction mix was incubated at 25°C for 5 min (DNA digestion) and 75°C for 5 min (DNAse inactivation). Then, each DNAase-treated RNA sample was reverse transcribed in a reaction consisting of: 16 µl DNAse-treated RNA template and 4 µl iScript Reverse Transcription Supermix in a final volume of 20 µl.

For intron-containing genes, specific qPCR primers, separated by at least one intron, were designed in order to avoid genomic DNA contamination. In this case, 1 µg of total RNA was reverse transcribed using iScript cDNA Synthesis kit (1708891; Bio-Rad Laboratories Inc., USA). Each RT mixture consisted of: 1 µg RNA, 4 µl 5x iScript Reaction Mix and 1 µl iScript Reverse Transcripase in a final volumen of 20 µl.

In both cases (intronless or intron-containing genes) the RT reaction mix was incubated in a thermal cycler (CFX96™ Real-Time PCR; Bio-Rad, Hercules, CA, USA) using the following protocol: 5 min at 25°C (priming), 20 min at 46°C (RT) and 1 min at 95°C (RT inactivation). Finally, the samples were diluted with Nuclease free water in a final volumen of 100µl and stored at -20°C.

Real time PCR for mRNA quantification*:* For real time PCR, we used Go Taq qPCR Master mix (A6102; Promega Corporation, USA) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., USA). PCR reactions were performed in duplicates for each experimental sample and consisted of: 5 µl cDNA, 0.5 µl specific primer forward (fw) 10nm, 0.5 µl specific primer reverse (rv) 10nm, 6.25 µl Go Taq qPCR Master mix and 2.75 µl of Nuclease free water (Final volume: 15 µl). Expression of each target gene was quantified using specific primers pairs (Table 1).

For data analysis, relative standard curves were constructed from serial dilutions of reference cDNA samples from selected tissues, including ovary or hypothalamus, and the input value of each target gene was standardized to the levels of (i) the ribosomal protein S11 (Rp-s11) or the hypoxanthine-guanine phosphoribosyltransferase (Hprt) for expression studies involving hypothalamic samples;

and (ii) Rp-s11 for expression studies involving ovarian samples. Primer-specific amplification and quantification cycles were run as follows: 1 cycle of Hot-Start activation at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s; annealing at specific temperature (see Table 1) for 30 s; extension at 72°C for 20 s and, a final extension of 72 °C for 10 min. The dissociation (Melting) curve was used to assess the quality of the PCR. Specificity of PCR products was confirmed by direct sequencing (Central Sequencing Service, Cordoba University). No-template controls were included in all assays.

Gene	Accession Number	Primers	Amplicon Size	Tissue	Melting Tº
Ngf	NM_001277055	Sen:5′-GGCCCATGGTACAATCTCCTTCAA-3′ Anti:5′-GTCCGTGGCTGTGGTCTTATCTCC-3′	136 pb	Ovary	63ºC
Ngfr	NM_012610.2	Sen:5′-AGCCAACCAGACCGTGTGTGA-3′ Anti:5′-GTCCTGGCAGGAGAACACGAG-3′	247 pb	Ovary	63ºC
Mkrn3	XM_218735.10	Sen:5′-AGTTGGACGAAGCAAATCCTC-3′ Anti:5′-AGGTCGTGAGAGTAGCGACA-3′	83 pb	Hypothalamus	60ºC
Kiss1	NM_181692.1	Sen:5´-GCTGCTGCTTCTCCTCTGTG -3´ Anti:5´-GCATACCGCGGGCCCTTTT -3′	138 pb	Hypothalamus	66ºC
Rp-s11	NM_031110.1	Sen:5′- CATTCAGACGGAGCGTGCTTAC-3′ Anti:5´- TGCATCTTCATCTTCGTCAC-3′	240 pb	Hypothalamus	58ºC
Hprt	NM_012583.2	Sen: 5′-AGCCGACCGGTTCTGTCAT-3′ Anti: 5′-GGTCATAACCTGGTTCATCATCAC-3′	72 pb	Hypothalamus	60ºC

Table 1: Primers list used for PCR analyses.

Calculation of expression levels of each target was conducted based on the cycle threshold (C_T) method. The C_T for each sample was calculated using the iCycler iQ real time PCR detection system software with an automatic fluorescence threshold (R_n) setting. The C_Ts from each sample was referred to the standard curve to estimate the mRNA content/sample, and the values obtained were normalized for procedural losses using Rp-s11 and Hprt mRNA (for hypothalamic tissues) or Rp-s11 (for ovarian tissues). The slope of the standard curve, which is represented as a semi-log regression line plot of C_T value vs. log of input nucleic acid, was used to estimate the PCR amplification efficiency.

Reverse transcription for miRNA*:* 10 ng of total RNA was reverse transcribed using TaqMan® MicroRNA Reverse Transcription Kit (4366596; Applied Biosystems™, USA). Each RT reaction consisted of: 10 ng total RNA diluted in 5 µl of Nuclease-free water, 3 µl 5X RT primer, 0.15 µl 100

mM dNTPs (with dTTP), 1 µl MultiScribe reverse transcriptase (50U/1µl) 1.50 µl 10X reverse transcription buffer, 0.19 µl RNase inhibitor (20U/µl) and 4.16 µl Nuclease-free water (final volume: 15 µl). This reaction mix was incubated in a thermal cycler (GeneAmp 9700) using the following protocol: 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Finally, the samples were diluted in a final volume of 215 µl and stored at -20°C.

Real time PCR miRNA quantification*:* Quantitative RT-PCRs were performed using predesigned assays for mir-30b (Assay Name: hsa-miR-30b; Ref: 000602, Applied Biosystems™, USA) and RNU6 (Assay Name: U6 snRNA; Ref: NR_004394, Applied Biosystems™, USA). Each PCR reaction consisted of: 1.33 µl of RT product (dilution 1:15), 1 µl of TaqMan® Small RNA Assay 20X, 10 µl of TaqMan® 2X Universal PCR Master Mix (No AmpErase®UNG), 7.67 µl of Nuclease-free water (final volume: 20 µl). The thermal cycling conditions were as follows: 2 min at 50°C, 10 min at 95°C (enzime activation), followed by 40 cycles of 15 s at 95°C (denaturation) and 1 min at 60°C (annealing) (Roche Light Cycler 480). Calculation of expression levels of each target was conducted as previously described for mRNA expression analyses. RNU6 served as the internal reference.

IN SITU HYBRIDIZATION ASSAYS

For analysis of the neuroanatomical distribution of Kiss1 mRNA expression in the hypothalamus, brains were collected from selected experimental groups and ISH analyses were performed, following previous protocols[159, 262]. Five sets of coronal 20 µm-thick sections were generated and mounted on SuperFrost Plus slides (Thermo Fisher Scientific Inc.). Standard procedures of tissue collection were applied, starting on a fixed coordinate in the rostral hypothalamic area up to ARC, to encompass relevant hypothalamic areas for the central control of puberty. The samples were stored at -80°C until used for ISH analyses.

For detection of Kiss1 mRNA by in situ hybridization, specific riboprobes spanning 83-371 nt of the rat Kiss1 mRNA sequence (GenBank NM_181692.1) were generated. First, a DNA template was synthesized by PCR using specific primers for Kiss1 cDNA amplification carrying at their 5'-end sequences for synthetic promoters for bacteriophage-encoded DNA-dependent RNA polymerases (T7 and T3). Primer sequences were as follows: T3-Kiss1 sense (5'-CAGAGATGCAATTAACCCTCACTAAAGGGA GATGGTGAACCCTGAACCCACA-3') and T7-Kiss1 anti-sense (5'-CCAAGCCTTCTAATACGACTCACTATAGGG AGAACCTGCCTCCTGCCGTAGCG-3'). For PCR reactions, Go Taq flexi DNA polymerase (Promega Biotech) was used, following the recommendations of the manufacturer. Reactions were performed in an iCycler (Bio-Rad Laboratories Inc.) using the following protocol: cDNA was denatured for 5 min at 95°C, and then 4 cycles were performed at 94°C for 1 min, 54°C for 2 min and 72°C for 30 s, followed by 35 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 30 s. A final extension at 72°C for 5 min was included. After electrophoresis on a 2% agarose (w/v) gel, a single DNA fragment of the expected size was obtained for each target and gel purified with a QiaQuick gel extraction kit (Qiagen). For the

generation of Kiss1 antisense riboprobe, the transcription reaction was as follow: 250 Ci [^{33}P]-UTP (Perkin Elmer), 0.5 µg of PCR product, 2 µl dNTPs (5 mM rATP, rCTP and rGTP), 1 µl RNasin Ribonuclease Inhibitor (Promega), 4 µl transcription buffer, and 2 µl T7 RNA polymerase (Promega) in a final volume of 20 µl. After 120 min of incubation at 37°C, one additional µl of T7 RNA was added to the mix, and the reaction was maintained for an additional 60 min more at 37°C. At the end, the residual DNA was digested with 2U DNase (Promega), and the reaction was stopped by addition of 3 µl 0.5 M EDTA pH 8.0. DEPC water was added to a final volume of 50 µl, and the labeled riboprobe was purified using illustra ProbeQuant G-50 Micro Columns (GE Healthcare). For the synthesis of Kiss1 sense riboprobes, the same procedure was applied, using T3 RNA polymerase (Promega).

A single set of sections was used for ISH (adjacent sections 100-µm apart). These tissue sections were: (i) fixed in 4% PFA for 15 min; (ii) stabilized with 0.1 M phosphate buffer (pH 7.4) at room temperature for 10 min; (iii) treated with saline triethanolamine and acetic anhydride to prevent non-specific binding of probes; (iv) washed in 2 µl saline-sodium citrate (SSC) buffer for 3 min; (v) dehydrated in increasing concentrations of ethanol; (vi) de-lipidated with chloroform; and (vii) air dried at room temperature for 1h. After these steps, hybridizations with Kiss1 riboprobe was performed for 16 h at 55°C. The stock hybridization buffer contained: 25 ml deionized formamide, 10 ml dextran sulfate 50%, 3 ml NaCl 5 M, 0.4 ml Tris base 1M (pH 8), 0.08 ml 0.5M EDTA (pH 8), 1X Denhardt's solution and RNase-free water up to 40 ml. Kiss1 riboprobe was added to the hybridization buffer to a final concentration of 0.03 pmol/ml along with yeast tRNA. After hybridization, slides were: (i) washed with 4X SSC for 30 min; (ii) incubated in RNase-A buffer (Roche Biochemical, USA) at 37°C (32 mg/ml) for 1 h; (iii) equilibrated with 2X SSC for 30 min; (iv) washed in 0.1X SSC for 1 h at 65°C; (v) dehydrated in increasing ethanol series; and (vi) air dried at room temperature for 1 h. Finally, slides were dipped in Kodak Autoradiography Emulsion type NTB (Eastman Kodak) and exposed for 1 week at 4°C in the dark. After this, the sections were developed and fixed following the manufacturer instructions (Kodak): (i) 4 min in Kodak Developer D-19; (ii) 10 s in distilled water; (iii) 5 min in Kodak Fixer; and (iv) 5 min in distilled water. For mounting, the sections were previously dehydrated and rinsed with Sub-X Clearing Medium (Leica Bio-systems). Then, slices were cover-slipped with Sub-X mounting medium (Leica Bio-systems). For analysis, 50-60 sections from each animal (9-10 slides; 6 sections/slide) were evaluated. Five animals per group were included in the analysis. Slides were read under dark-field illumination with custom-designed software enabled to count the total number of cells (grain clusters). Cells were counted as Kiss1 mRNA positive when the number of silver grains in a cluster exceeded that of background.

WESTERN-BLOT

Total protein was extracted from the whole hypothalamus, as previously described (see *"Samples*

collection" section). Briefly, total protein lysates (40 µg) were subjected to SDS-PAGE on 7% polyacrylamide gels, electro-transferred on polyvinylidene difluoride (PVDF) membranes (Millipore) and probed overnight at 4°C in the presence of the primary antibody anti-MKRN3 (1:500; Rabbit Anti-MKRN3 / RNF63 Polyclonal Antibody, Origene). For protein detection, we used horseradish peroxidase-conjugated secondary antibodies and chemi-luminescence (Abcam). 4-6 samples per group were assayed; protein levels were normalized to ß-actin (1:5000 dilution, A5060, Sigma Aldrich). Densitometic analysis of protein bands was conducted using the open source image processing software, ImageJ (https:// imagej.net/ImageJ).

IMMUNOHISTOCHEMISTRY

Brain preparation*:* Peripubertal or infantile female rats were anesthetized with ketamine-xylazine and perfused intracardially with saline (0.9% NaCl) followed by 4% PFA in PBS (pH 7.4). Fixed brains were immersed in 30% sucrose and 0.01% sodium azide in PBS at 4°C for 2-4 days. Next, 3 sets of coronal, 40 µm-thick sections were cut in a freezing microtome Leica CM1850 UV and stored at -20°C in cryo-protectant. For immunodetection of the different proteins, one set of sections encompassing the whole hypothalamus was used from each animal, and standard procedures for single-label immunohistochemistry were performed (see below).

Immunoenzymological staining*:* Brains from peripubertal or infantile female rats were assessed either for Kisspeptin, SPTLC1, and Mkrn3 immunolocalization. One set of free-floating brain sections from each animal was:

(i) Washed (3 x 10 min) in Tris-buffered saline (TBS) (pH 7.6) at room temperature with gentle agitation.
(ii) Blocked for endogenous peroxidases for 10 min.
(iii) Washed (3 x 10 min) in TBS (pH 7.6) at room temperature.
(iv) Incubated with a primary rabbit polyclonal anti-Kisspeptin 1:10000 (AC-566, was a generous gift from Dr. Alain Caraty; Institut National de la Recherche Agronomique, Tours, France); SPTLC1 Rabbit Polyclonal antibody 1:5000 (Proteintech) or Rabbit Anti-MKRN3 / RNF63 Polyclonal Antibody 1:1000 (Origene) at 4°C for 72 h in incubation buffer (0,25% donkey serum and 0.3% Triton X-100 in TBS).
(v) Washed (3 x 10 min) in TBS (pH 7.6) at room temperature;
(vi) Incubated with a secondary biotinylated donkey anti-rabbit antibody 1:500 (JAC-711-066-152 Jackson Immunoresearch) at room temperature for 90 min.
(vii) Washed (3 x 10 min) in TBS (pH 7.6).
(viii) Incubated in A/B Vectastain Elite solution (VECTASTAIN® Elite® ABC Kit reagents; Vector Laboratories, Burlingame, CA, USA) at room temperature for 90 min.
(ix) Washed (3 x 5 min) in TBS (pH 7.6) and acetate buffer 0,1M (3 x 5 min).

(x) Incubated with glucose oxidase and diaminobenzidine-nickel (DAB/Ni) for 20 min at room temperature.
(xi) Washed in acetate buffer 0,1 M and TSB buffer.
(xii) Mounted on silane-coated slices, air dried, and dehydrated in ascending concentrations of alcohol (50, 70, 95, 100, 100) and xylene and coverslip using Eukitt mounting medium (MICROPTIC S.L., Barcelona).
(xiii) Immunoreactivity was visualized in a microscope Leica DM2500 using the lense 10X.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

This technique was used to determine ceramide concentrations in the hypothalamus, as well as norepinephrine (NE) and 3-Methoxy-4-Hydroxyphenylglycol (MHPG) content in both the celiac ganglion and the ovary.

For ceramide determination, the hypothalamus was weighed and homogenized in 400 µl PBS and then quantified via an API 3000 PE Sciex (Spectralab Scientific, Markham, Ontario, Canada) liquid chromatography-electrospray ionization tandem mass spectrometer in positive ionization mode. Concentrations were measured by multiple reaction monitoring experiments using N-heptadecanoyl-D-erythro-sphingosine (C17 ceramide) as internal standard (50 ng/ml). The method was linear over a range of 2-600 ng/ml.

For NE and MHPG determinations, half of the ovary or the complete celiac ganglion was weighed and homogenized in 160-200 µl perchloric acid buffer (PCA) 0,2 M. Then, these samples were centrifuged at 3.500g and 4ºC for 15 min and the supernatant was collected and stored at -80ºC for further processing. Before the assay, 100 µl aliquots of supernatant were mixed with 100 µl PCA 0,2 N for each sample. Then, these samples were filtered through a 13mm diameter sterile filter with a 0.22 µm pore size hydrophilic PVDF membrane (Millex™). The quantification of NE and MHPG concentrations was implemented with an Eicom ECD-700 Electrochemical Detector for HPLC. Briefly, 20 µl of filtered samples were injected to a constant flow pump Jasco PU-2089s plus coupled to digitizer card Jasco LC-NetII/ADC with a Kromasil column 100-3.5-C18 (AkzoNobel). For chromatogram integration, a software JASCO ChromPass Chromatography Data System v1.7.403.1. was used. The solution used as a mobile phase for the assays contained: NaH_2PO_4 0,1 M, sodium octil sulphate 0,14 mM, EDTA 0,02 and acetonitrile 1,5%, pH 2,6. The flow speed employed was 1ml/min. The potential of the working electrode was set at +750 mV to detect simultaneously NE and MHPG.

RADIOIMMUNOASSAY (RIA)

Serum LH and FSH levels were measured using RIA kits supplied by the National Institutes of Health (Dr. A. F. Parlow, National Hormone and Peptide Program, Torrance, CA). Hormonal

determinations were performed in duplicates. Rat LH-I-10 and FSH-I-9 were labeled with ^{125}I by the chloramine-T method, and hormone concentrations were expressed using reference preparations LH-RP-3 and FSH-RP-2 as standards. Intra- and inter-assay coefficients of variation were less than 8% and 10% for LH and 6% and 9% for FSH, respectively. The sensitivity of the assay was 5 pg/tube for LH and 20 pg/tube for FSH. Accuracy of hormone determinations was confirmed by assessment of rat serum samples of known concentrations (used as external controls).

GnRH levels in incubation media were determined using a RIA kit provided by Phoenix Pharmaceuticals (RK-040-02; Karlsruhe, Germany). Intra- and inter-assay coefficients of variation were 4,7% and 8.3%, respectively. The sensitivity of the assay was 4 pg/tube.

6. STATISTICAL ANALYSIS

Statistical analyses were performed using Prism software (Graphpad Prism version 6.0 for Macintosh, GraphPad Software, La Jolla, California, USA, www.graphpad.com). The differences between several groups were analyzed by one-way ANOVA followed by the Tukey or Student-Newman-Keuls multiple comparison test for unequal replications. When comparing the influence of two different independent variables, experimental groups were subjected to two-way ANOVA test followed by Sidak's multiple comparisons test. The Student's t test was used to compare two groups. Hormonal determinations were performed in duplicate with a minimal total number of 8-12 determinations per group. RNA analyses were performed in duplicate from at least 5 independent samples per group. ISH assays were conducted in sets of 5 animals per group. WB were assayed with at least 4-6 samples per group. IHC for quantification of Kisspeptin positive neurons and fibers was conducted with 4-5 animals per group. All data are expressed as the mean ± SEM for each group; a P value of < 0.05 was considered statistically significant.

7. EXPERIMENTAL DESIGN

PART I: CENTRAL CERAMIDE SIGNALING EXPERIMENTS

EXPERIMENTAL SET #1: ANALYSIS OF THE ROLE OF CENTRAL CERAMIDE SIGNALING IN THE CONTROL OF PUBERTY ONSET AND ITS ALTERATIONS IN OBESITY

Considering that hypothalamic ceramides has been recently proposed as putative mediators for the actions of leptin and ghrelin in the control of food intake and energy balance, and that those hormones are crucial for the metabolic control of puberty, in the first set of experiments, we explored the potential role of central ceramide signaling in the control of puberty and its alterations in conditions of obesity.

Experiment 1: Hypothalamic ceramide content in an animal model of precocious puberty linked to early overnutrition.

To address the potential role of hypothalamic ceramides in the state of precocious puberty linked to early overnutrition, we first measured the level of hypothalamic ceramides in an animal model of early overnutrition (ON) that have been shown to induce overweight and advancement of puberty onset[257]. To this end, female pups were cross fostered and raised in small litters (SL) of 4 pups/dam from PND1 onwards. After weaning, SL rats were fed a high-fat diet (HFD; Diet D12451, 41 45% fat content; Research Diets Inc., New Brunswick, New Jersey) (ON). Normal litters (NL) of 12 pups per dam and fed from weaning with a control diet (Diet D12450B, 10% fat content) served as a model of normal nutrition (NN). Body weight and VO were monitored daily from PND25 onwards in both experimental groups (n=12). All animals were euthanized and hypothalami excised and processed for ceramide determination at PND36.

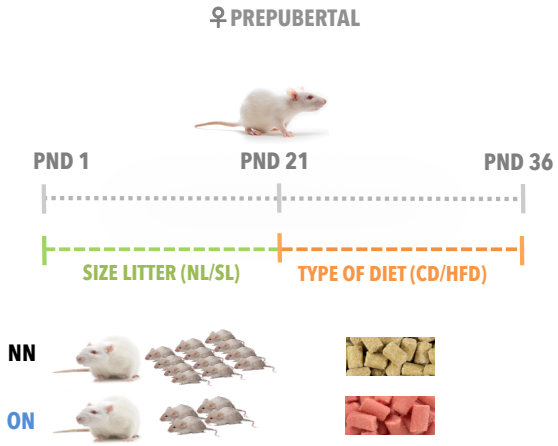


Figure 13: Schematic representation of the experiment 1.

Experiment 2: Manipulation of central ceramide signaling during pubertal development

We decided to assess the impact of pharmacological manipulation of central ceramide signaling on the timing of puberty in NN immature female rats. To this end, NN female rats received two daily icv injections with either CER C6 (2,5µg/12h/rat), a precursor of the synthesis of ceramides, or Myriocin, MYR (2µg/12h/rat), a specific inhibitor of the first enzyme of the *de novo* synthesis of ceramides, serine palmitoyltransferase, between PND26 and PND35. NN female rats icv injected with VEH (70% DMSO/12h/rat) served as controls. Body weight, food intake and VO were monitored daily between PND26 and PND35 in all experimental groups (n=12). On PND35, 60min after the last injection, animals were euthanized by decapitation and ovarian and uterus weights were recorded and blood, hypothalamus, and ovary samples were collected.

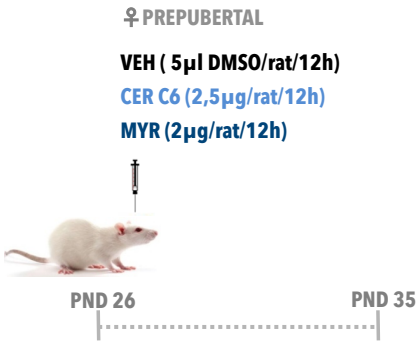


Figure 14: Schematic representation of the experiment 2.

EXPERIMENTAL SET #2: ANALYSIS OF THE NEUROENDOCRINE ROLE OF CERAMIDES IN THE CONTROL OF PUBERTY, INCLUDING THEIR INTERACTIONS WITH KISSPEPTIN AND LEPTIN, AND GnRH/ GONADOTROPIN REGULATORY ACTIONS

Based on the results of previous experiments, which suggested the impact of central ceramide signaling in the control of puberty, we decided to dissect out the potential mechanism(s) whereby hypothalamic ceramides may participate in those phenomena, with particular attention to their potential interplay with kisspeptin and leptin, as major regulatory signals of puberty. In addition, the putative role of ceramide signaling in the control of GnRH and gonadotropin secretion was explored in pubertal female rats.

Experiment 3: Exploring kisspeptin and leptin interactions with central ceramide signaling in the control of puberty.

To explore the potential role of central ceramide signaling in mediating leptin and kisspeptin effects on pubertal timing, we used an animal model of delayed puberty linked to chronic subnutrition. To this end, immature female rats were subjected from PND23 to PND36 to a moderate state of negative energy balance, imposed by 25% restriction of daily calorie intake (undernutrition; UN), as a means to decrease endogenous levels of leptin and hypothalamic mRNA content of Kiss1, in keeping with previous references[173]. UN rats received two daily icv injections of VEH (UN+VEH), kisspeptin (1nmol/12h/rat; UN+KP10), kisspeptin in combination with MYR (2µg/12h/rat; UN+KP-10+MYR), leptin (3.1µg/12h/rat; UN+LEP), or leptin in combination with MYR (2µg/12h/rat; UN+LEP+MYR), between PND29 and PND36. Animals fed ad libitum (NN) and injected with VEH served as controls (NN+VEH). Body weight and VO were daily monitored. On PND36, 60min after the last injection, animals were euthanized by decapitation, ovarian and uterus weights were recorded, and blood, hypothalamus, and ovary samples were collected.

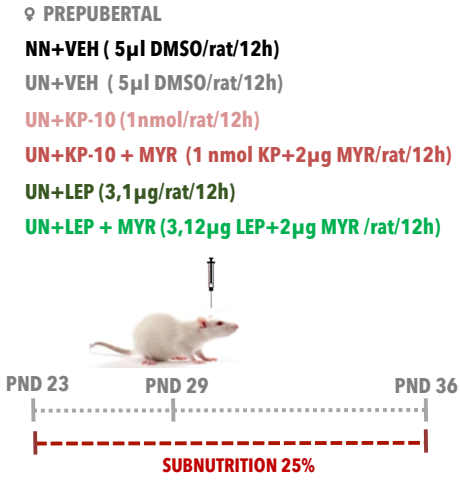


Figure 15: Schematic representation of the experiment 3.

Experiment 4: Analyses of hypothalamic Kiss1 mRNA expression after blockade of central ceramide signaling in prepubertal female rats.

The impact of blockade of central ceramide signaling on hypothalamic Kiss1 mRNA levels was assessed in prepubertal female rats. To avoid the potential confounding factor of changes in circulating estrogen on hypothalamic Kiss1 mRNA expression[116], female rats were subjected to bilateral ovariectomy (OVX) at PND25 and immediately supplemented with E2. E2 (10 mg/ml in olive oil) was provided in SILASTIC brand silicon tubing elastomers (Dow Corning, Midland, MI; 10 mm length; inner diameter, 0.062 cm; exterior diameter, 0.125 cm) and implanted subcutaneously, in line with previous references[159]. Subsequently, the animals received two daily icv injections of MYR (2µg/12h/rat) or VEH (70% DMSO/rat) between PND28 and PND32. On PND32, animals were euthanized, and brains were removed, frozen on dry ice and store at -80°C for further ISH analyses, as previously described.

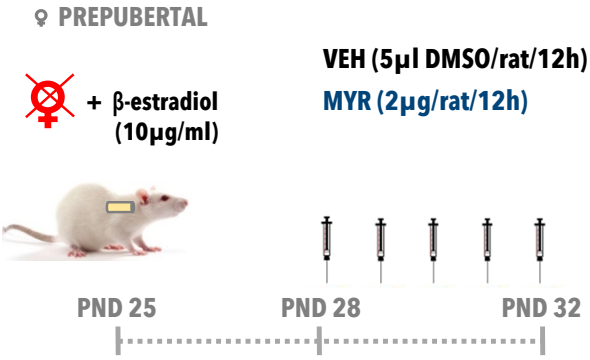


Figure 16: Schematic representation of the experiment 4.

Experiment 5: In vivo gonadotropin responses after blockade of central ceramide signaling, in combination with kisspeptin, in prepubertal female rats.

The effects of blockade of central ceramide signaling, in combination with kisspeptin, on prepubertal gonadotropin secretion were monitored in vivo. To this end, immature female rats were pre-icv injected with either VEH (70% DMSO/rat) or MYR (2μg/rat) on the evening of PND28 (8:00 pm) and the morning of PND29 (8:00 am). On PND29, 60 min after the last pre-icv injection, VEH animals received a single bolus of VEH or KP (1nmol/rat) and MYR pre-treated animals were icv injected with MYR or KP. Blood samples were collected at 15 min (jugular venipuncture) and 60 min (decapitation) after icv injections.

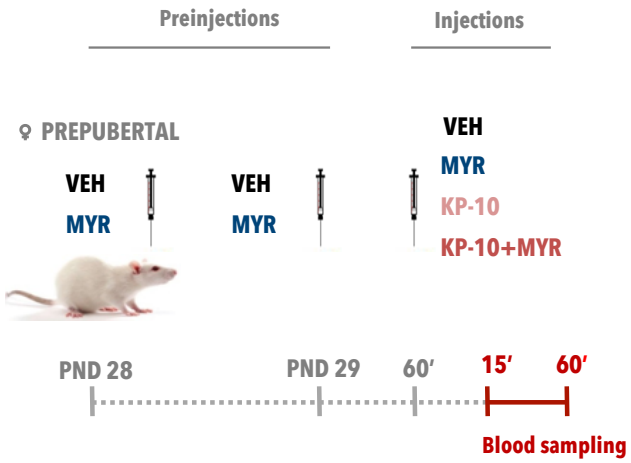


Figure 17: Schematic representation of the experiment 5.

Experiment 6: Ex vivo GnRH and gonadotropin responses to kisspeptin and GnRH, respectively, after manipulation of central ceramide signaling in prepubertal female rats.

The effects of manipulation of central ceramide signaling, in combination with kisspeptin, on prepubertal GnRH and gonadotropin secretion were monitored ex vivo. To this end, challenge of hypothalamic or pituitary explants from immature female rats (PND27-28) was conducted using a static incubation system, as previously described[258, 263, 264]. Briefly, upon decapitation of the animals, hypothalamic and pituitary tissues were removed and placed into individual incubation chambers in a Dubnoff shaker, at 37°C with constant shaking (60 cycles/min), under an atmosphere of 95% O_2–5% CO_2. Hypothalami were pre-incubated for 30 min in DMEM and further challenged for 45 min with CER C6 (10^{-7}M), MYR (10^{-7}M), KP (10^{-7}M) or a combination of KP (10^{-7} M) and MYR (10^{-7}M). Hypothalami pre-incubated with DMEM, and further incubated with fresh DMEM alone served as controls. At the end of the incubation period, medium samples were boiled for 30 min to inactivate endogenous protease activity and stored at −80°C until they were used for GnRH measurement. Pituitaries were pre-incubated for 60 min in DMEM and further challenged for 60 min with CER C6 (10^{-7}M), MYR (10^{-7}M), GnRH (10^{-8}M) or a combination of GnRH (10^{-8}M) and MYR (10^{-7}M). Pituitaries

pre-incubated with DMEM, and further incubated with fresh DMEM alone served as controls. At the end of the incubation period, medium samples were stored at −80°C until they were used for gonadotropin determinations.

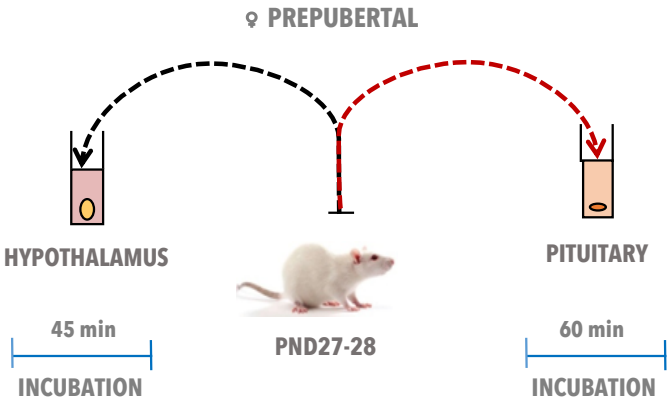


Figure 18: Schematic representation of the experiment 6.

EXPERIMENTAL SET #3: ANALYSIS OF A NOVEL CIRCUIT INVOLVING PVN-OVARIAN SYMPATHETIC INNERVATION FOR THE PUBERTAL ACTION OF THE KISSPEPTIN-CERAMIDE PATHWAY.

Because our previous experiments suggested that the effects of central ceramide signaling on the timing of puberty do not apparently involve alterations in GnRH/gonadotropin secretion, we decided to evaluate the putative role of an alternative ovarian sympathetic pathway in mediating the pubertal actions of hypothalamic ceramide signaling in murine models of precocious puberty linked to early overnutrition and normal pubertal development.

Previous data had suggested a potential role of the hypothalamic PVN as origin of the sympathetic innervation of the ovary, which appears to play a role in the modulation of pubertal timing. Considering in addition that one study has preliminarily reported the presence of kisspeptin fibers, with unknown function, in the PVN of adult female mice[265], in this set of experiments, we aimed to explore the potential pubertal roles of a pathway putatively involving PVN kisspeptin fibers/ceramide signaling and the ovarian sympathetic innervation in the control of puberty, and its alterations in conditions of precocious puberty linked to obesity.

Experiment 7: Impact of early onset obesity on the precocious activation of the ovarian sympathetic activity.

To explore the potential role of ovarian sympathetic tone in mediating central ceramide effects on precocious puberty linked to early overnutrition, we analyzed relevant markers of the ovarian sympathetic activity, including NE and its metabolite MHPG, as well as Ngf/Ngfr expression, in the celiac ganglia and/or the ovary of ON peripubertal female rats (ON). NN peripubertal female rats were

used as controls (n=10-12/group). Animals were euthanized specifically at PND25 to monitor the impact of early overfeeding on the ovarian sympathetic activity immediately prior to pubertal maturation. To this end, celiac ganglia and ovaries were collected for HPLC determinations of NE and MHPG and/or expression analysis of Ngf and Ngfr.

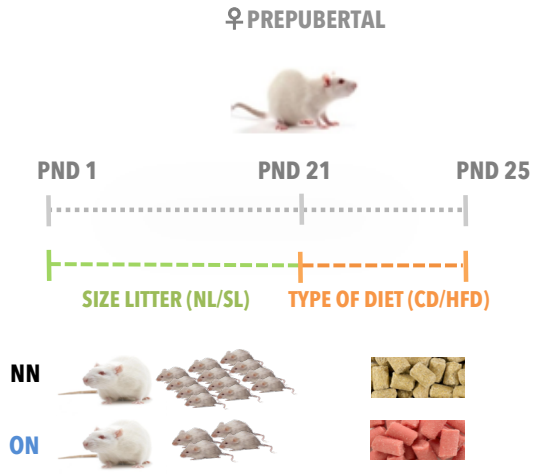


Figure 19: Schematic representation of the experiment 7.

Experiment 8: Impact of pharmacological blockade of central ceramide signaling on the timing of puberty and the ovarian sympathetic activity in an animal model of precocious puberty linked to early overnutrition.

Based on the high levels of hypothalamic ceramides and the early increase of ovarian sympathetic activity obtained in our animal model of precocious puberty linked to early overnutrition (*experiments 1 and 7*), we decided to evaluate the impact of blockade of central ceramide signaling on the timing of puberty and the ovarian sympathetic activity in this animal model. To this end, ON female rats received two daily icv injections with MYR (ON+MYR) at a dose of 2µg/12h/rat, between PND24 and PND29. NN and ON female rats icv injected with vehicle (NN+VEH and ON+VEH; 70% DMSO/12h/rat) served as controls. Body weight and VO were monitored daily between PND24 and PND29 in all experimental groups (n=12). In addition, ovarian and uterus weights were recorded, and blood, hypothalamus, celiac ganglion and ovary samples were collected after decapitation of the animals at PND29.

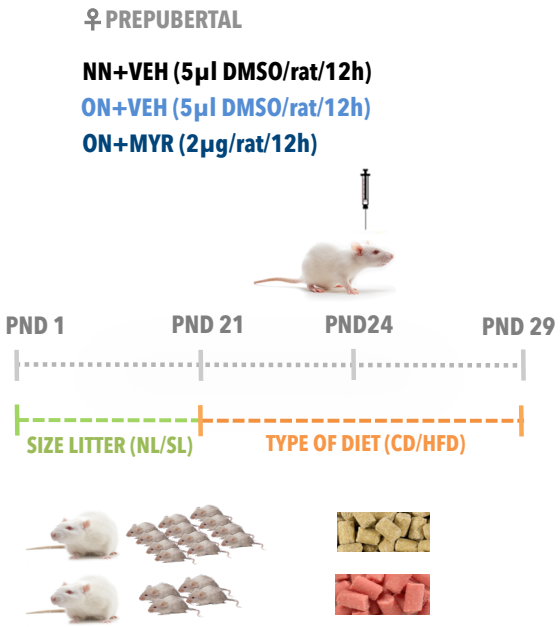


Figure 20: Schematic representation of the experiment 8.

Experiment 9: Quantitative analysis of PVN kisspeptin fibers and SPTLC1 content in an animal model of precocious puberty linked to early overnutrition.

The density of kisspeptin fibers and SPTLC1 at the PVN was analyzed in an animal model of precocious puberty linked to early overnutrition (PND29). To this end, ON female rats were anesthetized with ketamine-xylazine and perfused intracardially with saline (0.9% NaCl) followed by 4% PFA in PBS (pH 7.4) at the age when VO occurred. NN age-matched female rats served as controls. After perfusion, brains were collected and processed for IHC analyses at the PVN (*see "Immunohistochemistry" in "Material and methods"* section).

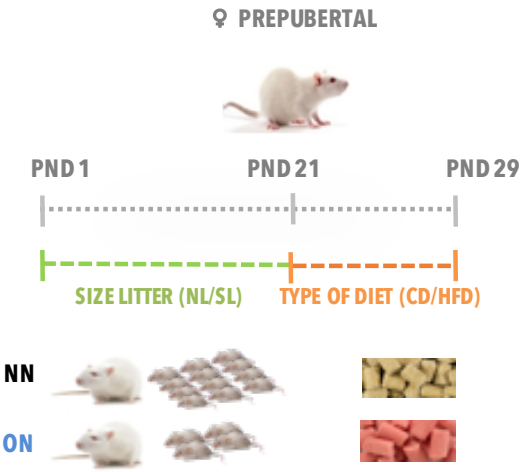


Figure 21: Schematic representation of the experiment 9.

PART II: miR-30b/Mkrn3 SYSTEM EXPERIMENTS

BIOINFORMATIC ANALYSES

In order to identify potential regulatory miRNAs of the Mkrn3 gene, we used four miRNA target prediction tools based on different methods (**Table 2**):

Method	Type of Method	Resource
miRanda [266]	Complementary	http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl
Target Scan [267]	Seed Complementary	http://www.targetscan.org
PicTar [268]	Thermodynamics	http://pictar.mdc-berlin.de/
MiRtaget2 [269]	Support Vector Machine	http://mirdb.org

Table 2: MicroRNA target prediction tools.

EXPERIMENTAL SET # 4: ANALYSES OF THE HYPOTHALAMIC EXPRESSION OF Mkrn3 AND miR-30b DURING NORMAL AND ALTERED PUBERTY AND NEUROANATOMICAL DISTRIBUTION.

The first set of experiments was focused on the analysis of the hypothalamic expression of Mkrn3 and miR-30b, for which our *in silico* analyses revealed the presence of three seed regions in a highly conserved area of the 3'-untranslated region (UTR) of Mkrn3, during normal pubertal development and in three different models of altered puberty induced by early developmental insults: neonatal estrogenization, photoperiodic manipulation and early postnatal underfeeding. In addition, the neuroanatomical distribution of Mkrn3 was also assessed at the infantile period.

Experiment 10: Hypothalamic expression of Mkrn3 and miR-30b during postnatal maturation.

The expression profiles of Mkrn3 mRNA and miR-30b, were determined in the hypothalamus of male and female rats at different age-points during postnatal maturation[46]: neonatal (PND1), late neonatal (PND7), infantile (PND15), juvenile (PND24 in females; PND30 in males), pubertal (PND36 in females; PND45 in males), and adult (PND-75) ages (n=5-9/group). The hypothalamus and/or medial basal hypothalamus (MBH) were collected, frozen on liquid nitrogen and stored at -80°C for further qPCR and/or WB analyses after decapitation of the animals.

Experiment 11: Hypothalamic expression of Mkrn3 and miR-30b in models of altered puberty.

To provide further evidence for the putative roles of this system in the maturational program leading to puberty, we conducted a comprehensive series of expression analyses in the following preclinical models of perturbed puberty:

Neonatal manipulation of sex steroids model: Neonatal male and female rats were exposed to high doses of estradiol benzoate (EB) as a model of disrupted brain sexual differentiation and altered puberty. As we previously mentioned, alterations of the sex steroid milieu during the critical neonatal period of sexual differentiation are known to disrupt pubertal maturation and gonadotropic function later in life[112]. Male and female rats (n=6/group) were injected subcutaneously (sc) on PND1 with olive oil alone (100 µl) (VEH: control group) or EB dissolved in olive oil (100 µg/rat in females; 500 µg/rat in males) as recommended[270].

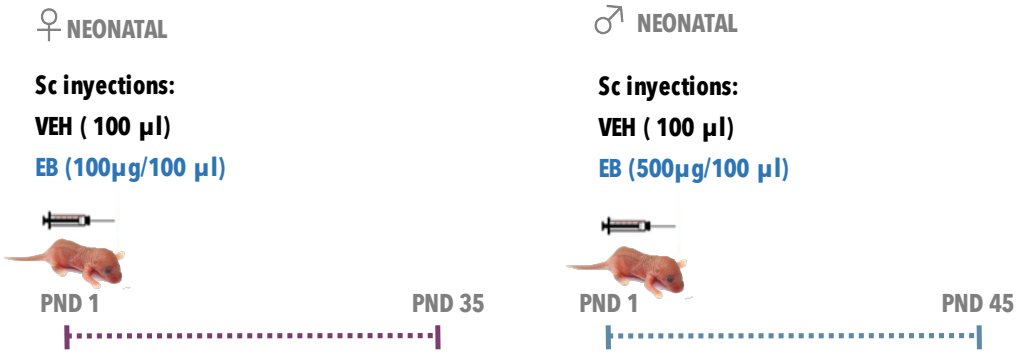


Figure 22: Schematic representation of neonatal manipulation of sex steroids model.

Photoperiodic manipulation model: Based on previous evidence showing that either changes in melatonin levels or photoperiod/day length modify the timing of puberty[271,272], a model of constant darkness (CD) during lactation, between PND5 and 10, was used. Subsets of animals (n=5) were euthanized and hypothalamic tissues collected at PND15 (i.e., immediately after completion of CD) and at puberty (PND35 in females; PND45 in males). Animals subjected to standard photoperiodic conditions (14h of light, from 7:00 am) were used as controls.

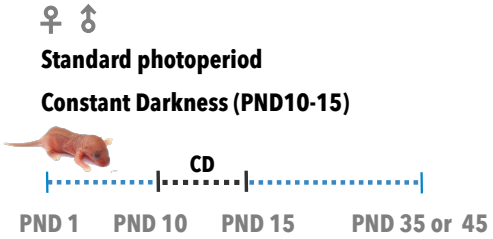


Figure 23: Schematic representation of photoperiodic manipulation model.

Early postnatal undernutrition model: Female rats bred in large litters (LL; 20 pups/dam) were used as model of delayed puberty[161]. This model has been proposed to mimic nutritional challenges during the last trimester of human gestation[273]. After weaning, the rats were reared with ad libitum access to water and food. Subsets of rats were sacrificed at PND5, 15, and 35 (females) or 45 (males). Animals bred in normal litters (NL; 12 pups/dam) were used as controls.

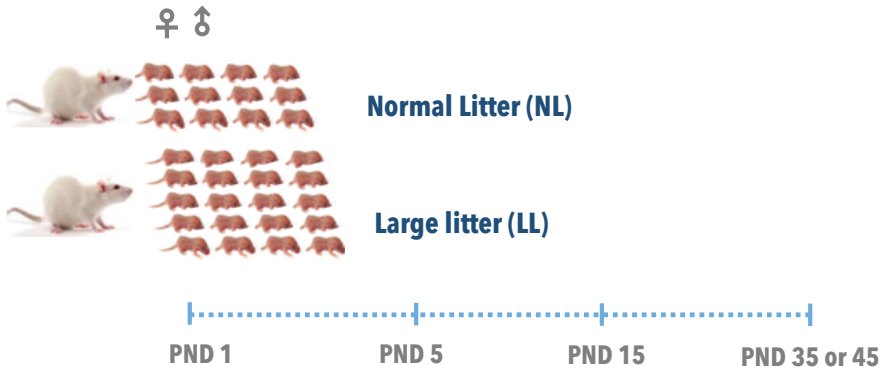


Figure 24: Schematic representation of early postnatal undernutrition model.

In all experimental models, hypothalami were collected, frozen on liquid nitrogen and stored at -80ºC for further qPCR and/or WB analyses after decapitation of the animals.

EXPERIMENTAL SET # 5: ANALYSIS OF THE FUNCTIONAL ROLE OF THE miR-30b/Mkrn3 PATHWAY IN THE CENTRAL CONTROL OF PUBERTY.

The next set of experiments aimed to provide functional evidence for the potential role of miR-30b/Mkrn3 pathway in the central control of puberty by using both in vitro and in vivo experimental approaches.

Experiment 12: Assessment of the repressive action of miR-30b on Mkrn3 expression in vitro.

A Luc-Pair miR luciferase assay was used to demonstrate targeting of the mouse Mkrn3 3′-UTR by miR-30b. Mkrn3 3′-UTR luciferase reporter construct (217MmiT030494-MT06), precursor expression plasmid for mmu-miR-30b (217MmiR3458-MR04), control 3′-UTR expression vector (217CmiT000001-MT06) and precursor miRNA scrambled control plasmid (217CmiR0001-MR04) were all obtained from Genecopoeia (Rockville, MD, USA). An empty expression vector, pcDNA3.1, kindly provided by Dr. M.A. Calzado (GC-04 group, IMIBIC, Cordoba), was also used in the assays. Renilla luciferase is included on the 3′-UTR Mkrn3 construct and was used to normalize for transfection efficiency.

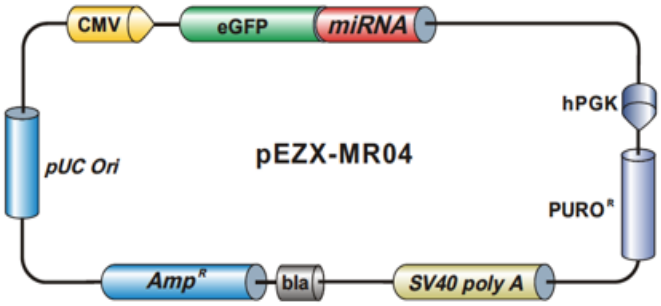


Figure 25: Precursor miRNA expression clone for mmu-mir-30b (pEZX-MR04 vector; *drawing taken from Genecopoeia*).

HEK-293T cells were used for the luciferase reporter experiment. The cells were maintained in DMEM (high glucose, Gibco, Life Technologies, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% (v/v) penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO2. For the assays, the cells were seeded onto 24-well plates ($1x10^5$ células/well) in DMEM containing 10% FBS. After 24 hours, cells were transiently co-transfected with (i) Mkrn3 3′-UTR-luc reporter and pcDNA3.1, (ii) Mkrn3 3′-UTR-luc reporter and scrambled miRNA vector, (iii) 3′-UTR control vector and mmu-miR-30b expression vector, and (iv) Mkrn3 3′-UTR-luc reporter and mmu-miR-30b expression vector at a concentration ratio 1:4 (250ng:1000ng), respectively. Transfections were performed using three replicates for each experimental condition. Polyethylenimine (PEI, #23966 Polysciences) was used as a transfection reagent at a concentration ratio 1 µg DNA: 3 µl PEI. After 5 hours of transfection, the medium containing transfection reagents was replaced by fresh DMEM; 24 hours later, the Renilla and Firefly luciferase activities were quantified by the Luc-Pair miR luciferase assay kit (217LPFR-M010, Genecopoeia, Rockville, USA) and measured on an automatic tube luminometer AutoLumat LB9510 (Berthold Technologies GmbH, Germany). Expression of the 3′-UTR reporter was measured by Firefly luciferase luminescence and normalized using the control Renilla-luc luminescence.

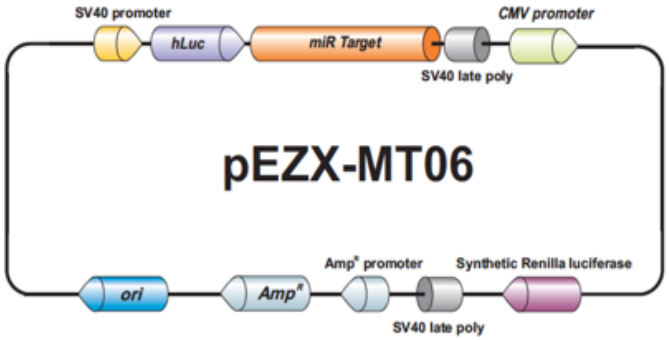


Figure 26: Mkrn3 Mouse miTarget expressing vector (217MmiT030494-MT06; *drawing taken from Genecopoeia*).

Experiment 13: Intracerebroventricular administration of selected target-site blockers against the binding sites of miR-30b in the 3'-UTR of Mkrn3 in immature female rats

To provide in vivo evidence for the functional role of the miR-30b/Mkrn3 pathway in the central control of puberty, we selectively prevented the binding of miR-30b to the 3'-UTR of the hypothalamic Mkrn3 gene during the juvenile-pubertal transition. To this end, we icv injected custom-designed miRNA target site blockers (TSB; miRCURY LNA™ microRNA Power Target Site Blocker in vivo use; Exiqon) in immature female rats. These miRNA TSBs are antisense-modified oligonucleotides that bind to the miRNA target site(s) of an mRNA and prevent miRNAs from gaining access to that site. Of note, these miRNA TSBs do not catalyze RNase H-dependent degradation of the mRNA. Therefore, miRNA TSBs administration would avoid the (mostly, repressive) actions of the miRNA, thereby inducing increased expression of the protein encoded by the targeted mRNA. miRNA TSB are designed to cover a larger sequence than the miRNA binding site to ensure target specificity. Three sequences were generated to protect the three miR-30b binding sites in the Mkrn3 3'-UTR (TSB-miR-30b). They were mixed at equimolar ratios (300pmol/5ul), and icv injected during two windows of postnatal development: (i) prepubertal treatment: TSB-miR-30b was icv injected at PND 24, 28 and 32; and (ii) juvenile treatment: TSB-miR-30b was icv injected at PND15, 22, 25 and 28. Body weight, food intake, VO and first estrus were daily monitored in both treatments. In addition, ovarian and uterus weights were recorded, and blood, hypothalamus, and ovary samples were collected after the decapitation of the animals (PND33 for prepubertal treatment and PND34 for juvenile treatment). Effectiveness of TSB-miR-30b treatment was validated by WB analyses of hypothalamic samples.

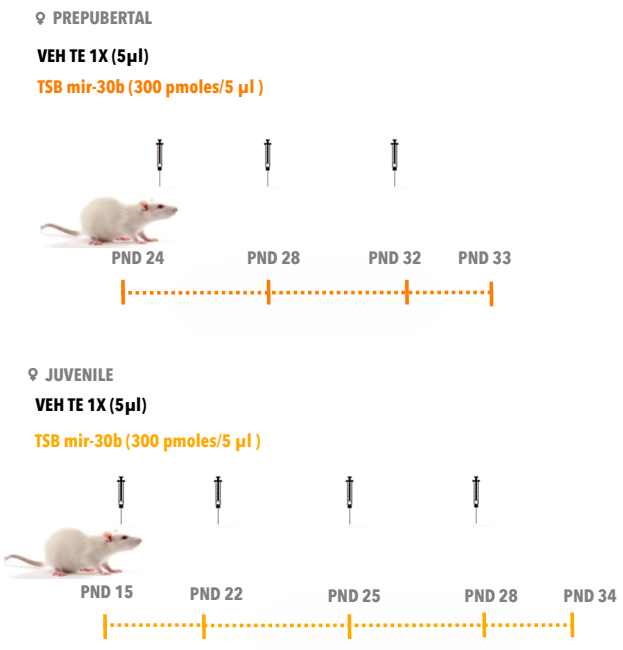


Figure 27: Schematic representation of the prepubertal (top) and juvenile (bottom) TSB-miR30b treatment

RESULTS

RESULTS

PART I: CENTRAL CERAMIDE SIGNALING & PUBERTY

HYPOTHALAMIC CERAMIDE CONTENT IS INCREASED IN OBESITY-INDUCED PRECOCIOUS PUBERTY

To explore the potential role of central ceramide signaling in the pathophysiology of precocious puberty linked to early overnutrition, we first analyzed the hypothalamic levels of ceramides in pubertal female rats subjected to early overnutrition (ON); a model that have been shown to induce early obesity and precocious puberty[257]. In line with previous references[257], ON female rats showed a higher BW (>20%) than their corresponding controls (NN; normonutrition group) at weaning, and such difference was manteined along pubertal development. In addition, ON female rats exhibited an earlier puberty onset, as measured by the age of vaginal opening (VO) and first estrus (**Figure 28**). Of note, the hypothalamic levels of total ceramides were significantly increased in ON animals with precocious puberty (**Figure 28**). These ceramides included 12 different types: CER C14, CER C16, CER C16:1, CER C18, CER C18:1, CER C20, CER C20:1, CER C22, CER C22:1(a), CER C24,CER C24:1(a) and CER C24:2(a). All of them, with exception for CER C24, were significantly elevated. Among them, CERC16:1 was the most significantly increased and CERC18 was the most abundant type.

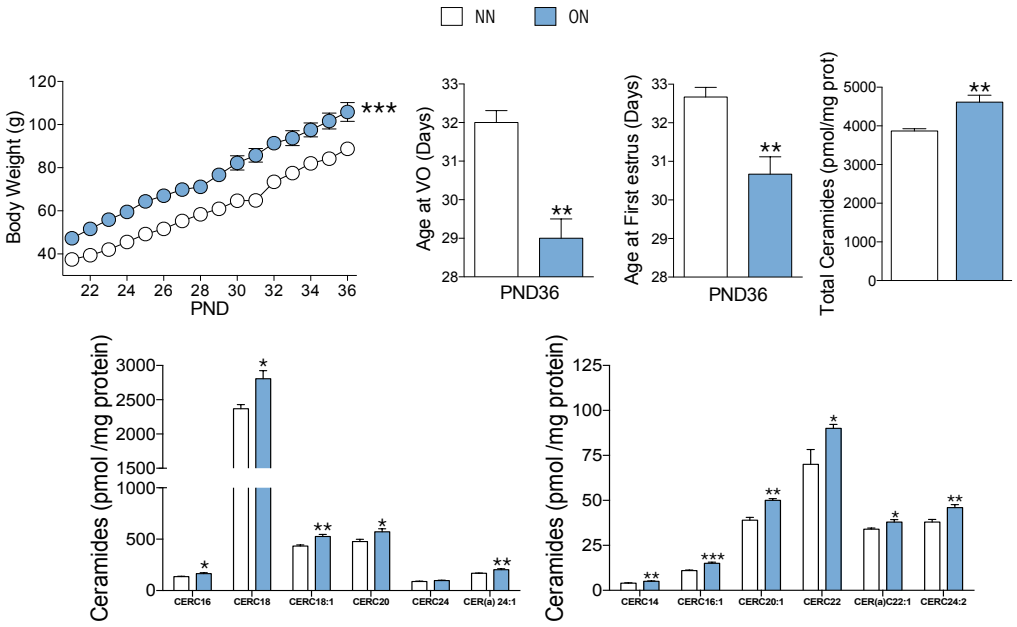


Figure 28: Evolution of body weight (BW) and mean of age at vaginal opening (VO) and first estrus of females rats subjected to early postnatal overnutrition followed by post-weaning HFD (ON) are presented. In addition, the hypothalamic levels of ceramides, which include 12 different species, of ON female rats and their corresponding controls (NN; normonutrition) are shown. Data are expressed as mean ± SEM (n=10-12 animals/group). *P≤0.05; **P≤0.01; **P≤0.0001 vs corresponding NN control group (Student t-test).

CENTRAL CERAMIDE SIGNALING IS INVOLVED IN THE CONTROL OF PUBERTY ONSET

To evaluate whether ceramides contribute to the central control of puberty onset during normal pubertal development, we analyzed the impact of pharmacological manipulations of hypothalamic ceramide signaling on the timing of puberty onset in immature female rats. Chronic stimulation of central ceramide synthesis by icv administration of CER C6, a cell-penetrating ceramide precursor, between PND26 and PND35 resulted in precocious puberty onset. This effect was evidenced by the earlier age of vaginal opening and first estrus observed in CER C6 animals (**Figure 29**), as well as the high number of CER C6 animals that underwent ovulation at PND35 as compared with their VEH control group (87% CER C6 vs. 62% VEH) (**Figure 30**). Of note, no alterations in BW, food intake (**Figure 29**) and relative ovarian and uterus weight (OW and UW) or gonadotropin levels were observed between CER C6 and VEH groups (**Figure 31**).

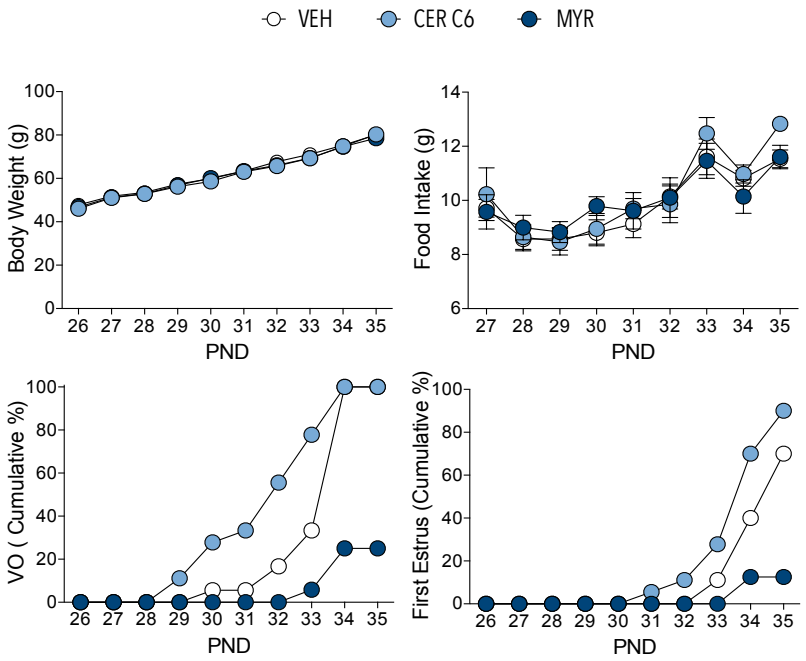


Figure 29: Impact of chronic manipulation of central ceramide signaling (PND26 to PND35) with CER C6, a precursor of ceramide synthesis, or MYR, an inhibitor of ceramide synthesis, on the evolution of body weight (BW), food intake, vaginal opening (VO) and first estrus in female rats during pubertal development. Females rats icv injected with vehicle served as controls (VEH) (n = 10-12 animals/group).

In contrast, persistent blockade of central ceramide synthesis by chronic icv treatment with MYR led to delayed vaginal opening and first estrus (**Figure 29**). In addition, central administration of MYR also reduced the number of animals that reached ovulation in relation to their VEH control group (10% MYR vs 62% VEH) (**Figure 30**). This phenotype was not linked to significant differences in BW, food intake (**Figure 29**), relative UW and gonadotropin levels between MYR and VEH groups

(**Figure 31**). Yet, a significant reduction in OW was detected in MYR animals. Together, these results demonstrate a novel role of central ceramide signalling in the control of puberty onset.

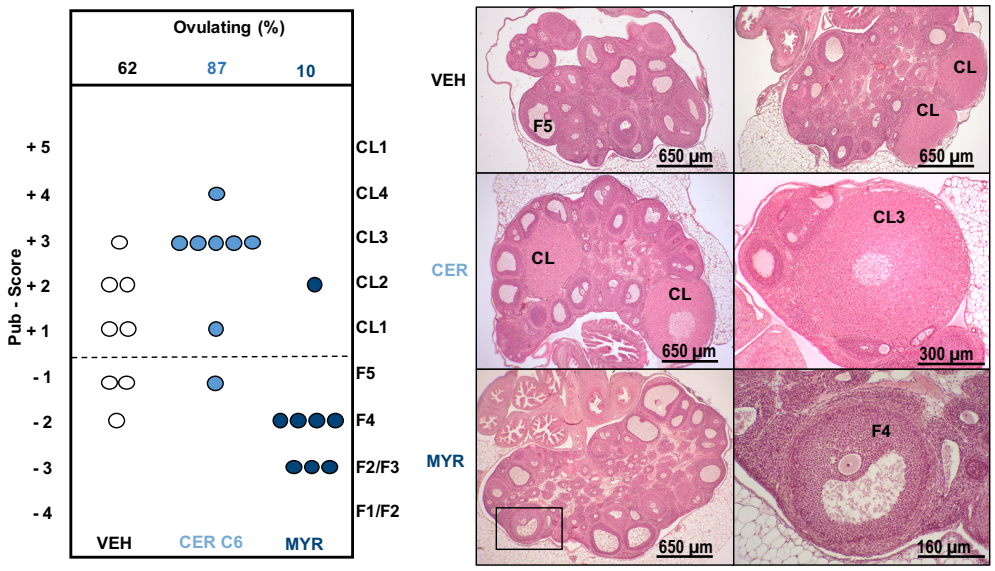


Figure 30: Pubertal ovarian maturation score (Pub-Score; left panel) and representative ovarian histological images (right panels) of Vehicle (VEH), Ceramide (CER C6) and Myriocin (MYR) treated animals. Pub-Score was established for each animal by evaluating the most advanced follicle stage for non-ovulating animals, and the age of the corpus luteum, as well as the most advanced follicle stage for already cycling animals. Non ovulating animals were scored from -4 to -1 when having follicle classes 1 to 5 as the most advanced stages. The day of ovulation was scored as 0. Ovulating animals were scored from +1 to +4 when having corpora lutea from 1 to 4 days of age (Left panel). F: Follicle; CL: Corporal lutea.

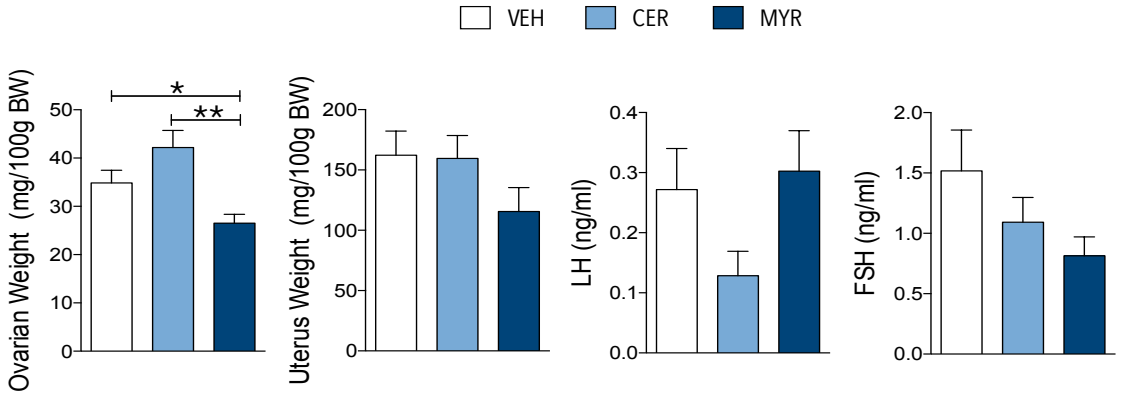


Figure 31: Impact of chronic manipulation of central ceramide signaling (PND26 to PND35) with CER C6, a precursor of ceramide synthesis, or MYR, an inhibitor of ceramide synthesis, on relative ovarian (OW) and uterus weight (UW), as well as LH and FSH levels in female rats at PND35. Females rats icv injected with vehicle served as controls (VEH). Data are expressed as mean ± SEM (n = 10-12 animals/group). *P≤0.05; **P≤0.01 (One-way-ANOVA followed by Student Newman Keuls multiple range test).

INTERACTION OF CENTRAL CERAMIDE SIGNALING WITH KISSPEPTIN AND LEPTIN IN THE CONTROL OF PUBERTY

To explore the potential mechanism(s) whereby central ceramides influence the timing of puberty, we first analyzed the potential interplay of hypothalamic ceramide signaling with two key modulators for the control of puberty onset, the puberty-stimulatory neuropeptide, kisspeptin, and the puberty-permissive hormone, leptin. To this end, we used a murine model of chronic moderate undernutrition (UN 25%) in which the hypothalamic content of kisspeptin and the endogenous levels of leptin are decreased, and the timing of puberty is delayed. This model allows (i) to detect the stimulatory/permissive actions of kisspeptin and leptin on the timing of puberty after their pharmacological replacement; and (i) to assess the impact of the blockade of central ceramide signaling on the pubertal actions of both kisspeptin and leptin in such context.

Food-restricted animals were subjected, from PND29 to PND36, to repeated icv injections of vehicle, kisspeptin or leptin, alone or in combination with the inhibitor of ceramide synthesis, MYR. Females fed ad libitum and chronically injected with vehicle served as controls. Chronic UN resulted in reduced BW (**Figure 32**), decreased relative UW and delayed puberty onset in peripubertal female rats icv injected with vehicle (UN+VEH group) (**Figure 33**). Yet, no changes in relative OW and LH levels were detected in those animals. In addition to induce a significant reduction in BW, administration of leptin to UN animals (UN+LEP group) was able to rescue the negative effect of subnutrition on pubertal timing, estimated by VO, and to induce a significant increase in relative UW. Similarly, kisspeptin treatment partially normalized the timing of puberty (>50%) and significantly enhanced LH secretion in UN animals (UN+KP group) (**Figure 33**).

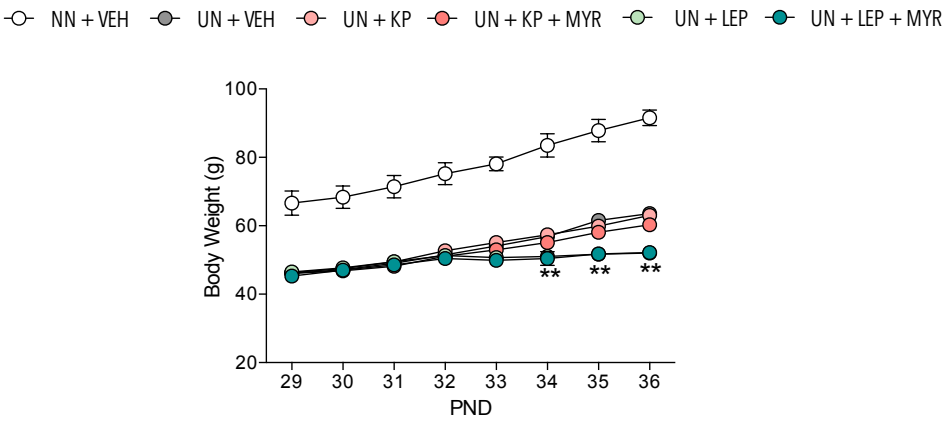


Figure 32: Evolution of BW during pubertal development in female rats subjected to 25% reduction in daily food intake (PND23 to PND36) and icv injected (PND29 to PND36) with vehicle (UN+VEH), kisspeptin (UN+KP) or leptin (UN+LEP), alone or in combination with the inhibitor of ceramide synthesis, MYR (UN+KP+MYR and UN+LEP+MYR). Females rats fed ad libitum and chronically injected with vehicle served as controls (NN+VEH). Data are presented as mean ± SEM (n = 8-11 animals/group). **P≤0.01 vs corresponding UN female rats (Two-way ANOVA followed by Sidak's multiple comparisons test).

Interestingly, concomitant blockade of central ceramide signaling with MYR largely prevented the stimulatory effects of kisspeptin on puberty onset in UN animals, as evidenced by delayed vaginal opening and decreased serum LH levels (UN+KP+MYR group). However, MYR co-administration only modestly suppressed the positive effect of leptin administration on the age of VO in UN animals (UN+LEP+MYR group) (**Figure 33**). Of note, MYR treatment was able to prevent the normalization in the age of first estrus induced by leptin treatment in UN animals (UN+LEP+MYR group; data not shown). Together, these results suggest that central ceramide signaling mediates the stimulatory/ permissive effects of KP and, to a lesser degree, LEP on puberty onset.

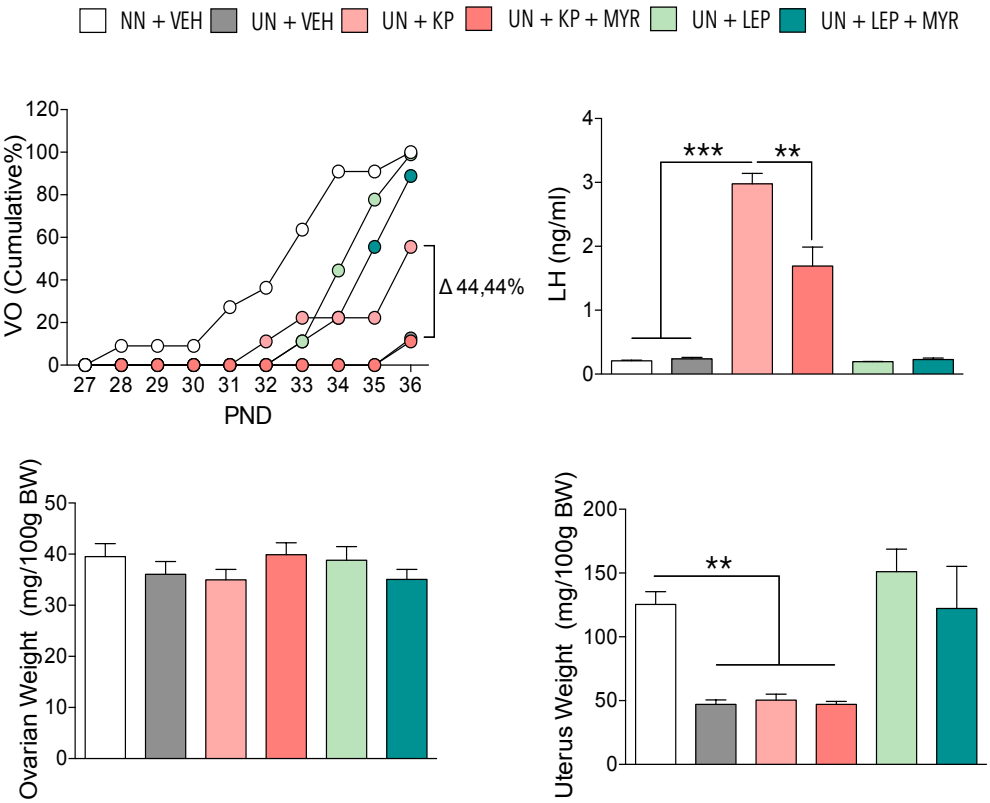


Figure 33: Impact of the chronic icv administration (PND29 to PND36) of the inhibitor of ceramide synthesis, MYR, in combination with kisspeptin or leptin on the timing of puberty onset in a model of chronic undernutrition (UN; 25% reduction in daily food intake from PND23 to PND36). In detail, the age of vaginal opening (VO) and LH concentrations (upper panels), as well as the relative ovarian weight (OW) and uterus weight (UW) (lower panels) from pubertal UN female rats treated with vehicle (UN+VEH), kisspeptin (UN+KP) or leptin (UN+LEP), alone or in combination with MYR (UN+KP+MYR and UN+LEP+MYR) are displayed. Females rats fed ad libitum and chronically injected with vehicle served as controls (NN+VEH). Results are represented as mean ± SEM (n=8-11 animals/group). **P≤0.01; ***P≤0.001 (One-way-ANOVA followed by Student Newman Keuls multiple range test).

BLOCKADE OF CERAMIDE SIGNALING DOES NOT ALTER HYPOTHALAMIC Kiss1 mRNA EXPRESSION AT PUBERTY

To evaluate the potential contribution of kisspeptin as central mediator for the neuroendocrine actions of ceramides in the control of puberty, we studied the impact of chronic blockade of central

ceramide signaling with MYR during the pubertal transition (PND28 to PND32) on hypothalamic Kiss1 mRNA expression, using in situ hybridization (ISH). As previously described (*see "Material and Methods"* section), we used OVX immature female rats supplemented with moderate doses of E_2 (i) to mimic physiological E_2 levels and (ii) to avoid the impact of fluctuating levels of endogenous estrogen on Kiss1 expression. In keeping with previous references[5,100], two main hypothalamic populations of Kiss1-expressing neurons were detected in the AVPV and the ARC. Chronic administration of MYR did not alter Kiss1 mRNA levels either in the AVPV or the ARC of peripubertal female rats **(Figure 34)**. These results support that the effects of central ceramide signaling on puberty onset are not likely mediated by primary alterations in the hypothalamic levels of Kiss1 mRNA.

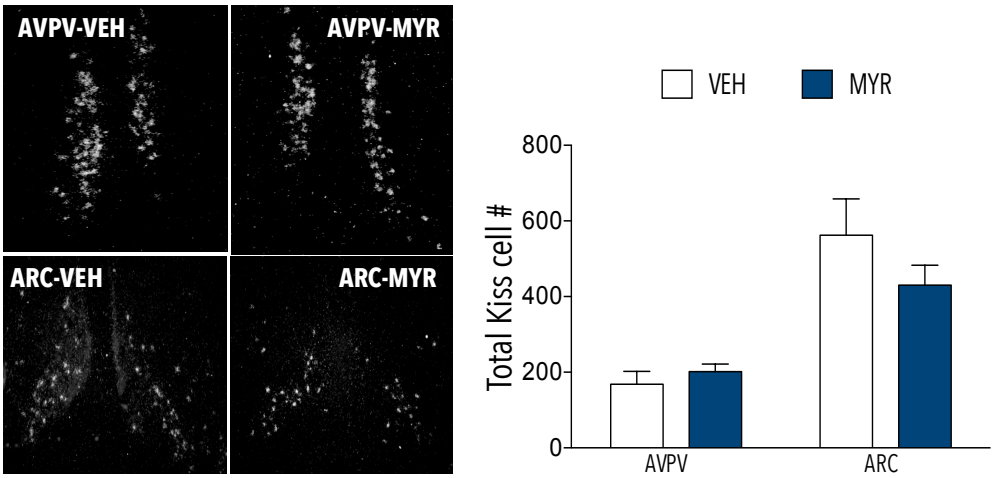


Figure 34: Impact of central ceramide inactivation on hypothalamic Kiss1 mRNA expression during pubertal development. The total number of Kiss1-expressing cells (defined as grain clusters) in the AVPV and ARC are represented (n = 5/group).

BLOCKADE OF CERAMIDE SIGNALING DOES NOT ALTER BASAL OR KISSPEPTIN-STIMULATED GnRH/GONADOTROPIN SECRETION

The potential interaction between kisspeptin and central ceramide signalling on pubertal GnRH and/or gonadotropin secretion was assessed using a combination of in vivo and ex vivo settings. In vivo, central pre-administration of two boluses of MYR did not affect either basal or kisspeptin-stimulated LH responses in prepubertal female rats, at 15 and 60 min post-kisspeptin injection (**Figure 35**).

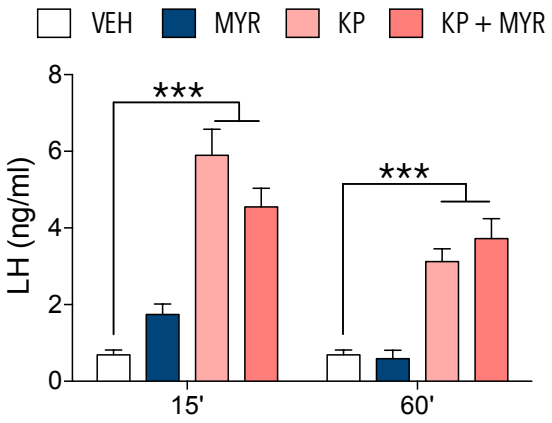


Figure 35: Effects of the central blockade of ceramide signaling with MYR in combination with kisspeptin on LH secretion in prepubertal female rats. Serum LH levels were measured in prepubertal female rats pre-icv treated with either vehicle or MYR (PND28 and PND29). Vehicle animals were later icv injected (PND29) with vehicle (VEH) or kisspeptin (KP) and MYR pre-treated animals were icv injected with MYR (MYR) or kisspeptin (KP+MYR). Ciculating LH concentrations at 15 and 60 min after the last icv injection are shown. Data are presented as mean ± SEM (n=10-12 animals/group). *** P ≤ 0.001 vs. corresponding VEH group at 15 and 60 min (One-way-ANOVA followed by Student Newman Keuls multiple range test.)

Ex vivo, challenge of hypothalamic explants from immature female rats with either CER C6 or MYR failed to alter GnRH secretory responses. Furthermore, MYR did not attenuate kisspeptin-induced GnRH release in this setting **(Figure 36)**.

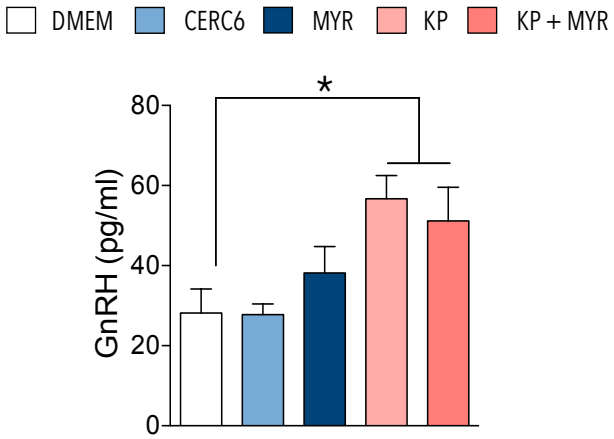


Figure 36: Effects of manipulation of central ceramide signaling and its interaction with kisspeptin on GnRH secretion in hypothalamic explants from immature female rats (PND27-28). GnRH levels from hypothalamic explants of inmature female rats incubated with the precursor of ceramide synthesis, CER C6, the inhibitor of ceramide synthesis, MYR, kisspeptin (KP) alone, or in combination with MYR (KP+MYR) are shown. Hypothalami incubated with medium (DMEM) served as controls. Data are presented as mean ± SEM (n = 10-12/group). *P≤0.05 vs DMEM (One-way-ANOVA followed by Student Newman Keuls multiple range test).

Finally, additional ex vivo experiments were performed to ascertain the role of ceramide signaling on gonadotropin release and its potential interplay with GnRH in such context. In line with the above results, the incubation of pituitary explants from prepubertal female rats with either CER C6 or MYR did not significantly modify gonadotropin secretory responses. Moreover, MYR did not prevent GnRH-

induced gonadotropin release in this setting **(Figure 37)**. Together, these data demonstrate that central ceramide signaling does not affect GnRH/gonadotropin secretion at puberty and that kisspeptin does not seem to mediate the potential neuroendocrine effects of central ceramide signaling on pubertal GnRH/gonadotropin release.

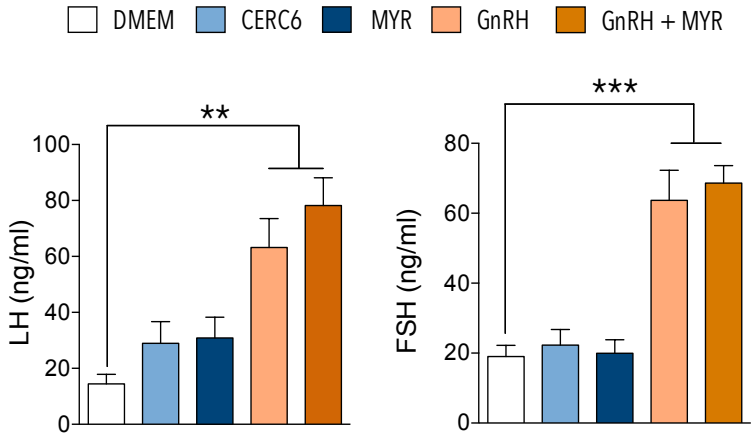


Figure 37: Effects of manipulation of ceramide signaling and its interaction with GnRH on LH secretion by pituitary explants from immature female rats (PND27-28). LH levels in incubation media of pituitary explants of inmature female rats incubated with the precursor of ceramide synthesis, CER C6, the inhibitor of ceramide synthesis, MYR, GnRH alone, or in combination with MYR (GnRH+MYR) are shown. Pituitaries incubated with medium (DMEM) alone served as controls. Data are presented as mean ± SEM (n = 10-12/group). **P≤0.01; ; ***P≤0.001 vs DMEM (One-way-ANOVA followed by Student Newman Keuls multiple range test).

A NOVEL CIRCUIT INVOLVING KISSPEPTIN-CERAMIDE SIGNALING AT PVN AND OVARIAN SYMPATHETIC INNERVATION IS ALTERED IN OBESITY-INDUCED PRECOCIOUS PUBERTY

In addition to the classic neuroendocrine regulation, substantial evidence supports that the ovarian function is also subjected to neural control[46,65]. In particular, the PVN has been proposed as the major hypothalamic region for transmitting sympathetic neural information to the ovary[66]. This sympathetic input is crucial for relevant aspects of ovarian maturation and function, including steroidogenesis and early follicular development[46,68]. Due to the lack of evidence supporting the impact of central ceramide signaling on GnRH/LH secretion, we decided to assess whether kisspeptin and central ceramide signaling interplay at an *alternative* PVN-ovarian sympathetic pathway that might be altered in obesity-induced precocious puberty.

Before analyzing the potential role of PVN kisspeptin-ceramide signaling and ovarian sympathetic activity in obesity-induced precocious puberty, we decided to specifically ascertain whether the ovarian sympathetic tone is altered in immature female rats subjected to early overnutrition (ON). To this end, relevant markers of ovarian sympathetic activity, such as NE and its metabolite, MHPG, were measured in the celiac ganglion, responsible for transmitting sympathetic inputs to the ovary, and the ovary of ON female rats at PND25. In addition, Ngf and Ngfr mRNA expression, as surrogate markers of the sympathetic tone of the ovary, were analyzed in those animals. We found that NE and

MHPG levels were significantly increased in the celiac ganglion and the ovary of ON female rats at PND25 as compared to their control lean counterparts (NN). Furthermore, such increase was also linked to higher levels of ovarian Ngf mRNA. Yet, no significant changes in ovarian Ngfr content were observed in ON groups, albeit a trend for increase in Ngfr expression was detected (**Figure 38**).

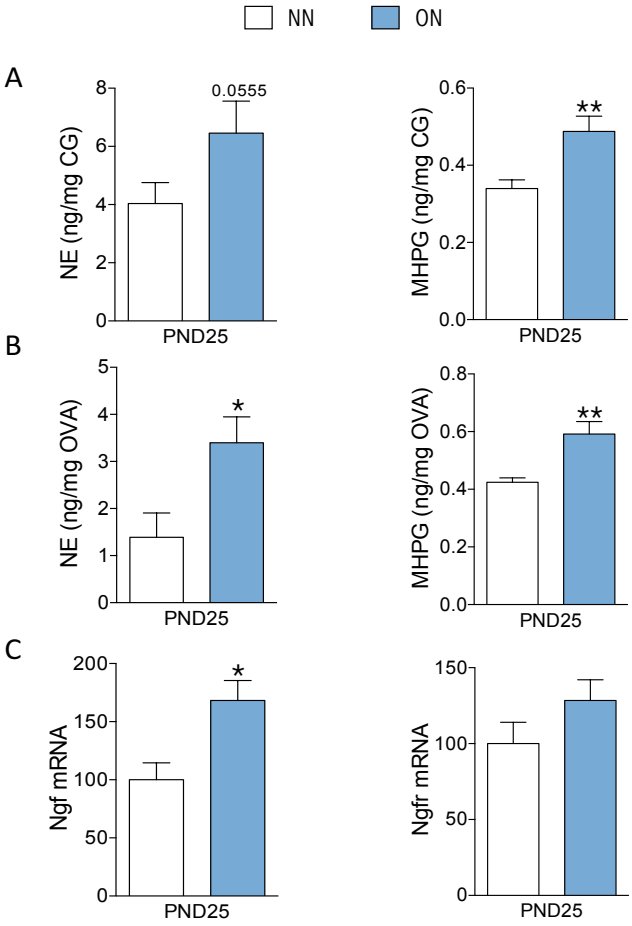


Figure 38: Impact of early overnutrition on the ovarian sympathetic tone in immature female rats (PND25). Levels of norepinephrine (NE) and its metabolite, MHPG (3-Methoxy-4-Hydroxyphenylglycol), in the celiac ganglion and the ovary of immature female rats (PND25) subjected to early overnutrition (ON) are shown (A, B). In addition, ovarian mRNA levels of Ngf and Ngfr in ON female rats (PND25) are represented (C). Immature female rats (PND25) bred in normal litters and subjected to post-weaning standard diet (NN) served as controls. Data are shown as the mean ± SEM (n = 6 animal/ group). *P≤0.05;**P≤0.01 (Student-t test).

To assess whether central ceramide signaling may underlie obesity-induced precocious puberty through alterations in the ovarian sympathetic activity, we analyzed the impact of the pharmacological inhibition of central ceramide signaling on the timing of puberty and the ovarian sympathetic tone in ON female rats. ON female rats chronically icv injected with VEH (ON+VEH) or the inhibitor of ceramide synthesis, MYR (ON+MYR), displayed a higher BW than their corresponding controls (NN+VEH) from the beginning of the treatment (PND24), and remained heavier up to puberty (PND29) (**Figure 39**).

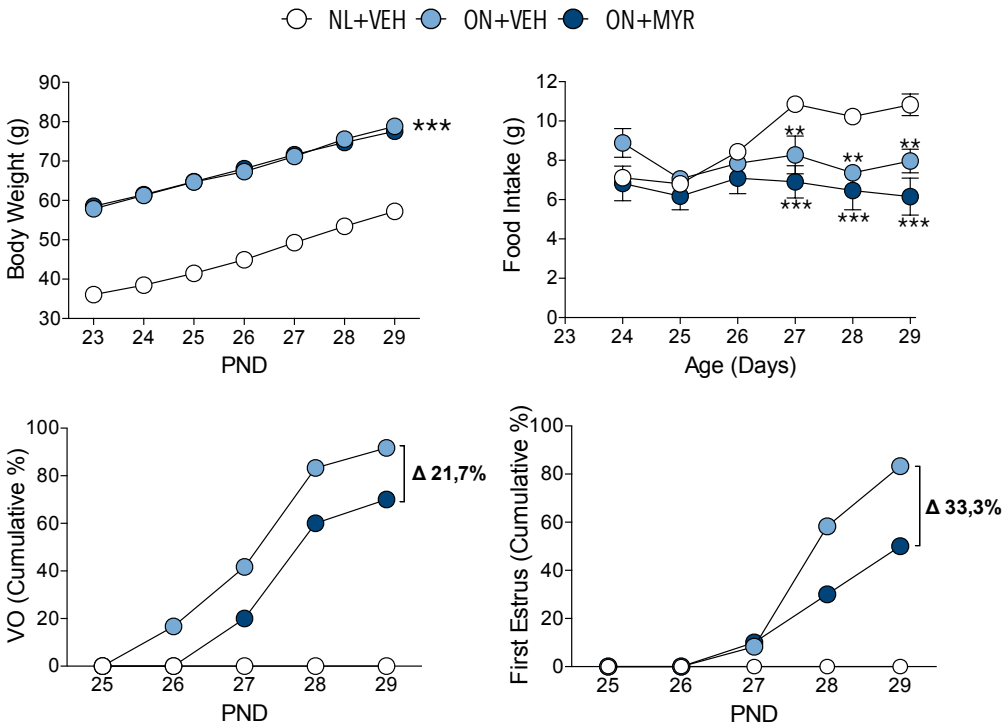


Figure 39: Impact of central blockade of ceramide signaling with MYR (PND24 to PND29) on the evolution of body weight (BW), food intake, vaginal opening (VO) and first estrus in female rats subjected to early ovenutrition (ON +MYR). ON and lean (NN; normonutrition) female rats icv injected with vehicle served as controls (ON+VEH and NN+VEH, respectively). Data are presented as mean ± SEM (n = 10-12 animals/group). **P≤0.01; ***P≤0.001 vs corresponding NN female rats (Two-way ANOVA followed by Sidak's multiple comparisons test).

Furthermore, ON+VEH female rats showed precocious puberty, as demonstrated by the earlier age of VO and first estrus (**Figure 39**), as well as the higher percentage of animals that underwent ovulation (**Figure 40**). In contrast, chronic blockade of central ceramide signaling with MYR in ON female rats (ON+MYR) partially normalized the phenotype of precocious puberty induced by early overnutrition. This phenomenon was evidenced by the partial delay in the age of VO and first estrus observed in ON+MYR animals as compared to ON+VEH group (VO, ON+MYR: 70% vs. ON+VEH: 91,7%; first estrus: ON+MYR: 50% vs. ON+VEH: 83,3%) (**Figure 39**), as well as the reduced number of ON+MYR animals that reached ovulation (50% ON+MYR vs. 83% ON+VEH) (**Figure 40**).

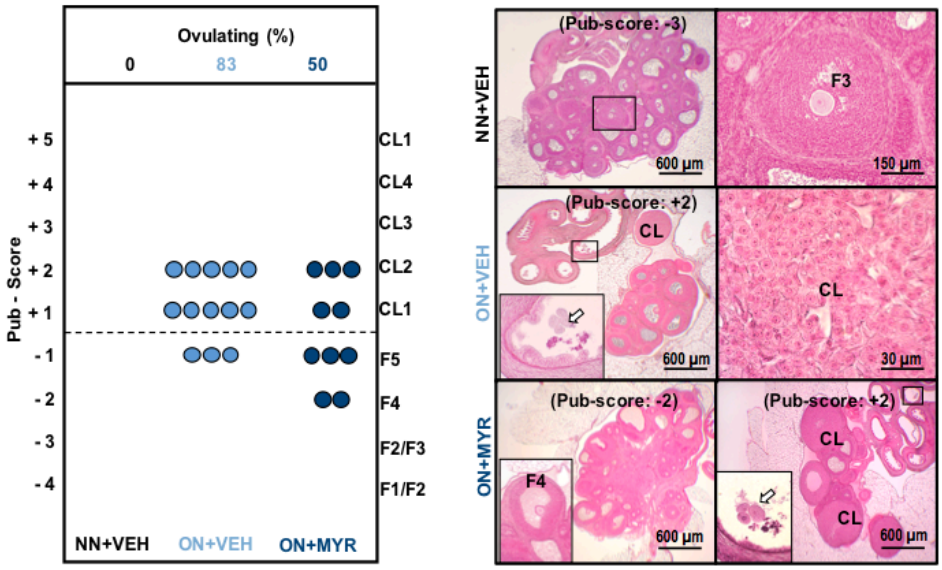


Figure 40: Pubertal ovarian maturation score (Pub-Score; left panel) and representative ovarian histological images (right panels) of lean female rats icv injected with vehicle (NN+VEH) and early overfed female rats icv treated with vehicle (ON+VEH) or MYR (ON+MYR). Pub-Score was established for each animal by evaluating the most advanced follicle stage for non-ovulating animals, and the age of the corpus luteum, as well as the most advanced follicle stage for already cycling animals. Non ovulating animals were scored from -4 to -1 when having follicle classes 1 to 5 as the most advanced stages. The day of ovulation was scored as 0. Ovulating animals were scored from +1 to +4 when having corpora lutea from 1 to 4 days of age (Left panel). F: Follicle; CL: Corporal lutea.

No significant changes in relevant metabolic factors, such as BW and food intake, or additional reproductive parameters, including relative OW and UW, as well as gonadotropin levels were observed between ON+VEH and ON+MYR groups. Yet, a significant increase in LH levels and relative UW was detected between NN+VEH and ON+VEH groups (**Figure 41**). Additionaly, it is worth to note that total food intake was apparently decreased in ON groups vs. NN group, regardless of the VEH or MYR, thus likely reflecting the satiety effect of the highly caloric HFD used in our experiments (**Figure 39**).

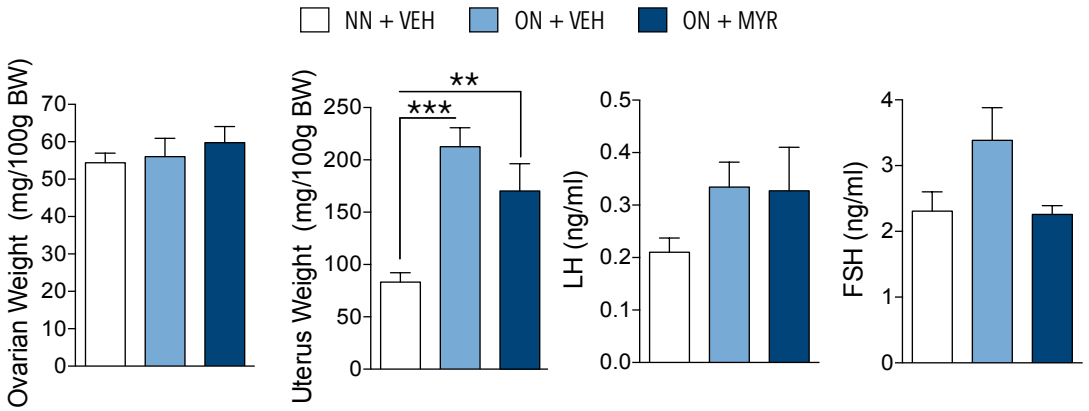


Figure 41: Impact of central blockade of ceramide signaling with MYR (PND24 to PND29) on relative ovarian (OW) and uterus weight (UW), as well as LH and FSH levels in female rats subjected to early ovenutrition at PND29 (ON+MYR). ON and lean (NN; normonutrition) female rats icv injected with vehicle served as controls (ON+VEH and NN+VEH, respectively). Data are expressed as mean ± SEM (n = 10-12 animals/group). **P≤0.01; ***P≤0.001 (One-way ANOVA followed by Student Newman Keuls multiple range test).

Interestingly, the above pubertal phenotypes were associated with significant changes in relevant markers of ovarian sympathetic activity. Thus, while a significant reduction in NE content was detected in the celiac ganglion and the ovary of ON+VEH female rats with precocious puberty, the levels of NE were normalized in the celiac ganglion and the ovary of ON+MYR female rats, together with a partial normalization of puberty (**Figure 42**). Furthermore, mRNA levels of Ngf and Ngfr, two additional markers of ovarian innervation and sympathetic activity, were significantly decreased in ON+MYR at puberty (PND29) (**Figure 42**). Together, these results suggest that perturbed central ceramide signaling might contribute to the pathophysiology of precocious puberty linked to early overfeeding through alterations in the ovarian sympathetic activity.

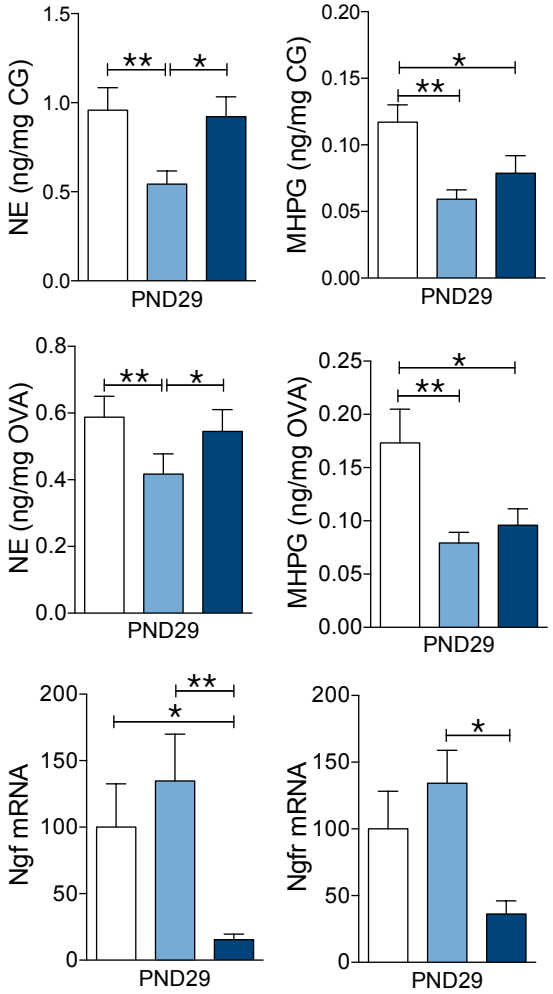


Figure 42: Impact of central blockade of ceramide signaling with MYR (PND24 to PND29) on the ovarian sympathetic activity of female rats subjected to early ovenutrition (bred in small litters and subjected to HFD after weaning) at PND29 (ON+MYR). Levels of norepinephrine (NE) and its metabolite, MHPG (3-Methoxy-4-Hydroxyphenylglycol), in the celiac ganglion and the ovary of ON+MYR female rats are shown. In addition, ovarian mRNA levels of Ngf and Ngfr in ON+MYR female rats are represented. ON and lean (NN; normonutrition) female rats icv injected with vehicle served as controls (ON+VEH and NN+VEH, respectively) (n = 10-12 animals/group). Data are shown as the mean ± SEM (n = 6 animal/group). *P≤0.05;**P≤0.01 (One-way-ANOVA followed by Student-Newman-Keuls multiple range test).

Finally, we evaluated the specific contribution of a putative interplay of Kisspeptin-central ceramide signaling at the PVN to the phenotype of precocious puberty induced by early overnutrition. To this end, we analyzed the presence and potential alterations of kisspeptin-ir and serine palmitoyltransferase long chain base subunit 1 (SPTLC1) expression in the PVN of early overfed female rats (ON) with precocious puberty; SPTLC1 is a key enzyme for the *de novo* ceramide synthesis.

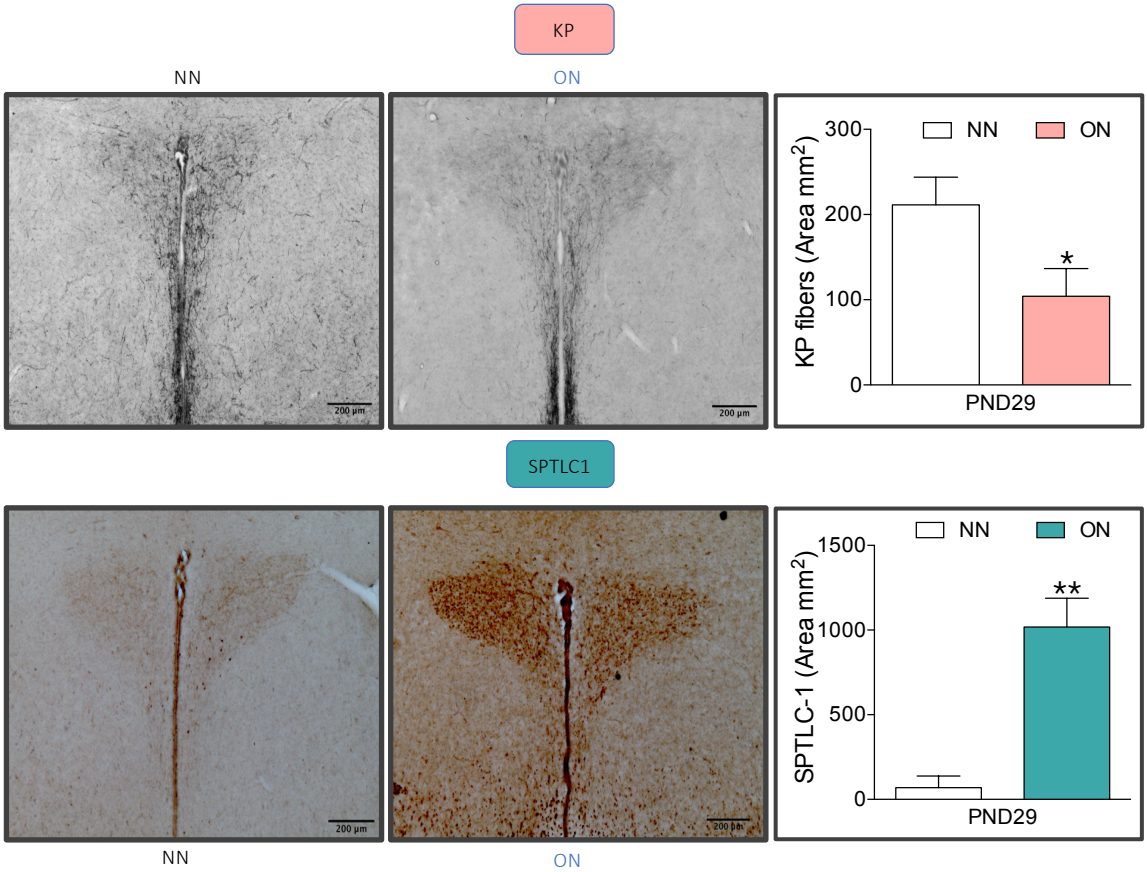


Figure 43: Representative photomicrographs and quantitative data from immunohistochemical analyses of kisspeptin and SPTLC1 (serine palmitoyltransferase long chain base subunit 1) immunoreactivity (ir) in the PVN of early overfed female rats at PND29. Peripubertal female rats (PND29) bred in normal litters and subjected to post-weaning standard diet (NN) served as controls . Data are shown as the mean ± SEM (n = 5-6 animals/group). *P≤0.05;**P≤0.01 (Student-t test). Scale bar, 200 µm.

Our results documented not only the presence of kisspeptin-ir and SPTLC1 expression in the PVN of ON female rats with early puberty but also changes in its patterns of immunoreactivity. Thus, a reduced kisspeptin-ir was detected in the PVN of ON female rats at PND29 as compared to their corresponding lean controls (NN). In contrast, a significant increase in the expression of SPTLC1 was observed in the PVN of those ON animals. Together, these results suggest that alterations in kisspeptin-ir and ceramide synthesis at the PVN might influence the ovarian sympathetic activity and underlying obesity-induced precocious puberty (**Figure 43**).

PART II: miR-30b/Mkrn3 SYSTEM & PUBERTY

IN SILICO IDENTIFICATION OF BINDING SITES FOR miR-30 FAMILY MEMBERS AT THE 3'-UTR OF Mkrn3 mRNA

Using four miRNA-target prediction tools based on different bioinformatic methods (see "*Material and Methods*" section), we obtained various sets of potential regulatory miRNAs for the Mkrn3 gene (**Table 3**). Interestingly, all of them highlighted the members of the miRNA family, miR-30, as strong candidates for the potential regulation of Mkrn3 gene. This is based on the top "score" obtained for all of them by using different miRNA-target prediction tools, and most importantly, the identification of the three predictive seed regions highly conserved among species detected for those miRNAs in the 3' untranslated region (UTR) of Mkrn3. In particular, three seed regions for 5 members of the miR-30 family (namely, miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e) are located in a highly conserved area that covers more than 200bp of the 3'-UTR of Mkrn3 (**Figure 44**).

A

MiRtaget2 score	miRNA Name
87	**rno-miR-30e-5p**
87	**rno-miR-30a-5p**
87	**rno-miR-30d-5p**
87	rno-miR-384-5p
87	**rno-miR-30b-5p**
87	**rno-miR-30c-5p**
87	rno-miR-495
86	rno-miR-217-5p
81	rno-miR-466b-2-3p
81	rno-miR-466b-4-3p

B

MiRanda Score	miRNA Name
182.015	**mmu-miR-466d-3p**
180.365	**mmu-miR-466a-3p**
178.165	**mmu-miR-467e***
177.164	**mmu-miR-30e**
177.164	**mmu-miR-30a**
17.316	**hsa-miR-548a-5p**
17.316	**mmu-miR-21***
170.157	**mmu-miR-297a***
169.403	**hsa-miR-922**
169.156	**mmu-miR-302c***
169.156	**mmu-miR-30d**
166.414	**mmu-miR-302b***
166.153	**mmu-miR-544**
166.147	**hsa-miR-768-3p**
165.153	**hsa-miR-570**
165.153	**mmu-miR-466h**
165.153	**mmu-miR-467a***
163.914	**mmu-miR-26b**
163.151	**mmu-miR-26a**
16.215	**hsa-miR-548c-5p**
16.215	**mmu-miR-495**
16.215	**mmu-miR-743b-3p**
161.431	**mmu-miR-384-5p**
161.149	**mmu-miR-30b**
161.149	**hsa-miR-519c-3p**
160.435	**mmu-miR-217**
160.435	**hsa-miR-602**
159.438	**mmu-miR-30c**
158.886	**mmu-miR-219**
158.765	**hsa-miR-641**

C

PicTar score	miRNA Name
8.72	hsa-miR-30a-5p
8.72	hsa-miR-30a-5p
8.72	hsa-miR-30a-5p
8.72	hsa-miR-30c
8.72	hsa-miR-30c
8.72	hsa-miR-30c
8.72	hsa-miR-30b
8.72	hsa-miR-30b
8.72	hsa-miR-30b
8.72	hsa-miR-30e
8.72	hsa-miR-30e
8.72	hsa-miR-30e
7.47	hsa-miR-30d
7.47	hsa-miR-30d
7.47	hsa-miR-30d
2.59	hsa-miR-124a
2.59	hsa-miR-124a
2.59	hsa-miR-124a

D

miRNA family	Conserved sites (TargetScan)				Poorly conserved sites (TargetScan)			
	Total	8mer	7mer-m8	7mer-A1	Total	8mer	7mer-m8	7mer-A1
miR-30abcde-5p/384-5p	3	3	0	0	0	0	0	0
miR-145-5p	0	0	0	0	1	**1**	0	0
miR-217-5p	0	0	0	0	1	**1**	0	0
miR-23-3p	0	0	0	0	2	0	**1**	**1**
miR-133-3p	0	0	0	0	1	0	0	**1**
miR-26-5p	0	0	0	0	1	0	**1**	0
miR-194-5p	0	0	0	0	1	0	0	**1**
miR-200bc-3p/429	0	0	0	0	1	0	**1**	0

Table 3. Potential miRNA regulators for Mkrn3 identified by different bioinformatics tools: MiRtaget2 (A), MiRanda (B), PicTar (C) in rat (rno), mouse (mmu) and human (hsa). In addition, the number of conserved binding sites for the potential miRNA candidates predicted by TargetScan (www.targetscan.org/) at the 3'-UTR of Mkrn3 transcript is shown (D). The best miRNAs candidates are highlighted in black.

Because of the relatively large number of miRNAs candidates, including prominently all members of miR-30 family, we decided to select one representative of this family for further studies. Among them, miR-30b was picked on the following basis: (i) its documented expression in rat hypothalamus[274]; (ii) its demonstrated regulation by sex steroids[275, 276]; and, (iii) the high number of predictive and conserved binding sites detected for this miRNA in the 3'-UTR of Mkrn3 (**Table 3** and **Figure 44**).

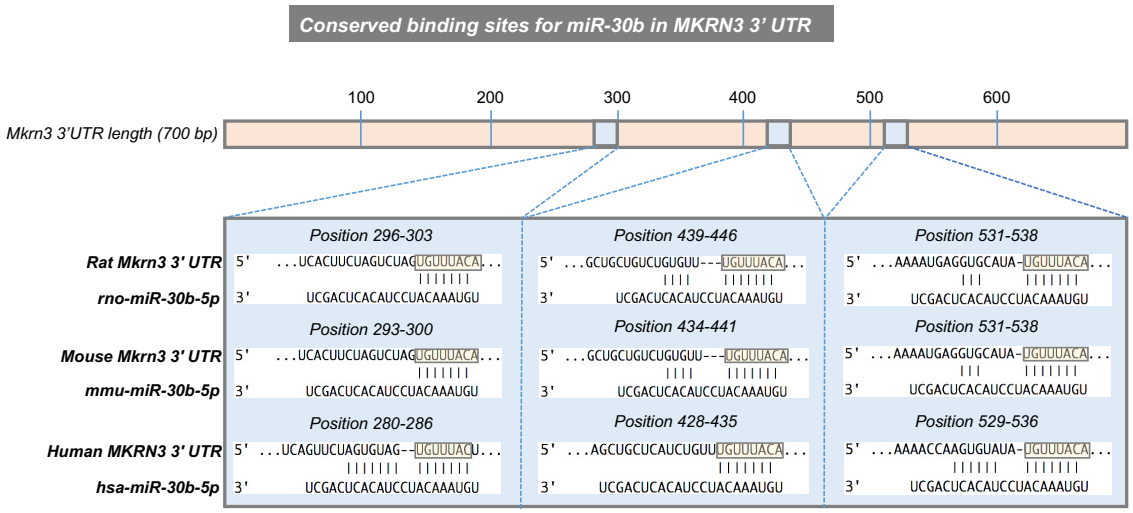


Figure 44: Schematic representation of predicted binding sites for miR-30b, a representative member of miR-30 family, in rat, mouse and human MKRN3 3'-UTR by using TargetScan (www.targetscan.org/). Consequential pairings of the target region (top) and miR-30b (bottom) are shown. Seed regions for miR-30b at the 3'–UTR of Mkrn3 are underlined in light orange.

OPPOSITE HYPOTHALAMIC EXPRESSION PROFILES OF Mkrn3 mRNA AND miR-30b DURING POSTNATAL MATURATION IN MALE AND FEMALE RATS

The expression of Mkrn3 mRNA progressively declined during postnatal development in the whole hypothalamus of both female and male rats. This decrease was particularly pronounced at the time of puberty in both sexes (PND35 in females and PND45 in males; **Figure 45**). Hypothalamic levels of miR-30b anti-paralleled those of Mkrn3, namely, low expression was detected during the infantile period, with a significant increase at pubertal ages, in both female and male rats (**Figure 45**). The analysis of the neuroanatomical distribution of Mkrn3 in infantile female rats (PND10; selected due to the high hypothalamic levels of Mkrn3), revealed abundant Mkrn3-immunoreactivity (ir) in the ARC (**Figure 46**), a relevant hypothalamic region for the control of puberty that is contained within the MBH. Based on the above data, further developmental qPCR analyses were specifically performed at the MBH of female and male rats, including additional postnatal ages.

Hypothalamus

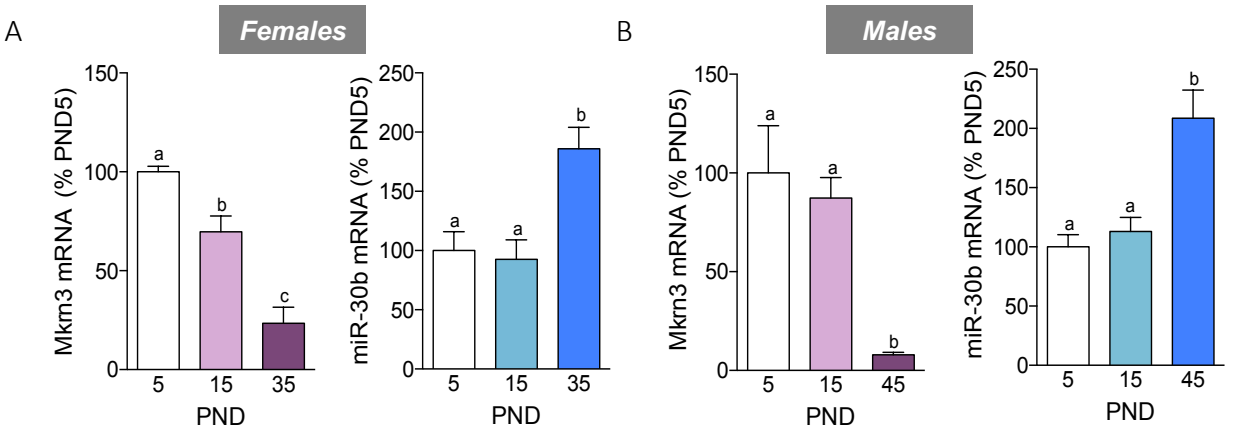


Figure 45: Expression profiles of Mkrn3 mRNA and miR-30b in the whole hypothalamus of female (A) and male (B) rats during postnatal maturation. Data are presented as mean ± SEM (n = 5-9 animals/group). Different superscript letters above bars indicate statistical differences (One way ANOVA followed by post hoc Tukey test).

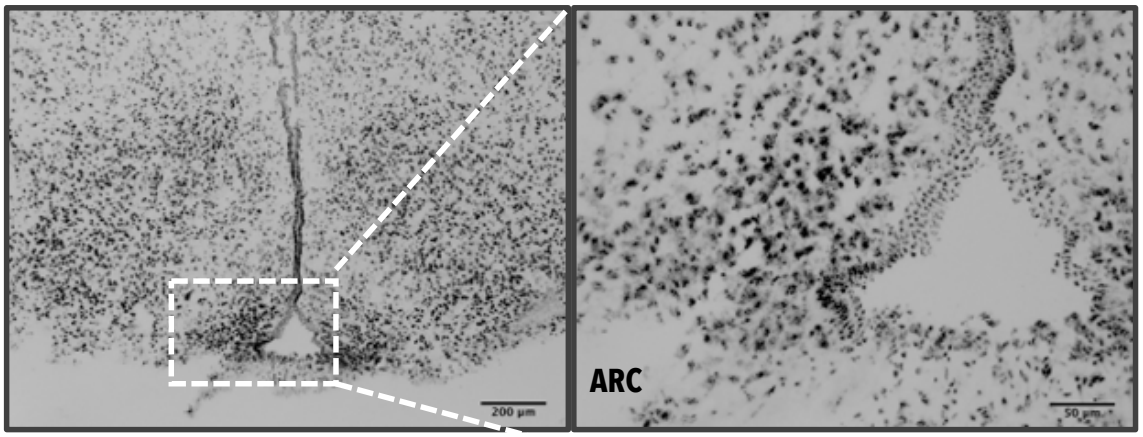


Figure 46: Representative photomicrographs of Mkrn3 immunoreactivity (ir) in the hypothalamus of infantile female rats (PND10) are shown. Specific neuroanatomical distribution of Mkrn3-ir in the hypothalamic ARC nuclei is presented at higher magnification (right panel). Scale bars, 200 µm and 50 µm.

In line with initial findings, similar expression patterns of Mkrn3 mRNA were detected in the MBH of both female and male rats during postnatal development. In particular, Mkrn3 displayed very high expression levels during the infantile period (PND 1-15 in both male and female rats), markedly declined during the infantile-juvenile transition (PND 15-24 in female rats and PND15-30 in male rats), reached minimal levels during the peripubertal period (PND 24-36 in female rats and PND 30-45 in male rats), and remained at low values throughout adulthood (**Figure 47**). In contrast, miR-30b levels were minimal neonatally and progressively increased during postnatal maturation, reaching maximum levels during the peripubertal period in both female and male rats (**Figure 47**).

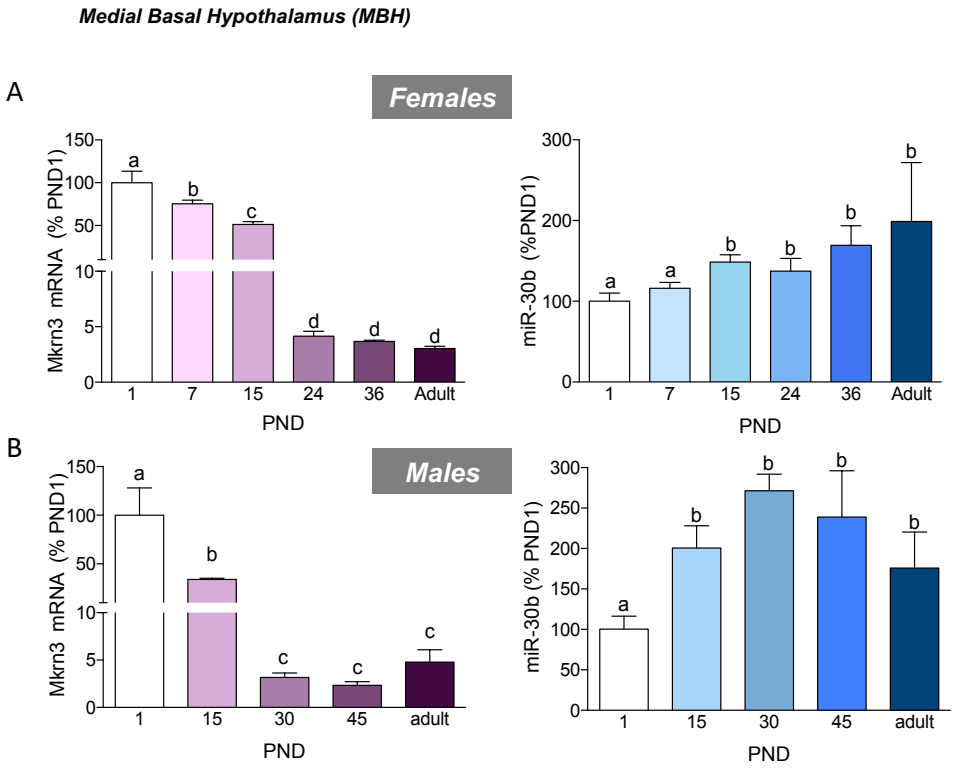


Figure 47: Expression profiles of Mkrn3 mRNA and miR-30b in the medial basal hypothalamus (MBH) of female (A) and male (B) rats during postnatal maturation. Data are presented as mean ± SEM (n = 5-9 animals/group). Different superscript letters above bars indicate statistical differences (One-way ANOVA followed by post hoc Tukey test.

ALTERED HYPOTHALAMIC EXPRESSION PROFILES OF Mkrn3 mRNA and miR-30b IN PRECLINICAL MODELS OF PERTURBED PUBERTY

Model of neonatal steroid manipulation: In agreement with previous publications[13], neonatal administration of high doses of EB altered pubertal maturation in both male and female rats, as evidenced by absent BPS or perturbed VO, respectively, and decreased serum gonadotropin levels in both sexes (data not shown). This phenotype was linked to changes in the hypothalamic expression of Mkrn3 mRNA and miR-30b in female rats at the expected time of puberty. Thus, on PND 35, neonatally estrogenized female rats displayed a significant increase in Mkrn3 mRNA content as compared with their controls (VEH). In contrast, miR-30b levels were significantly reduced in those animals (**Figure 48**). In male rats neonatally injected with EB, no alterations in the hypothalamic levels of Mkrn3 mRNA or miR-30b were detected at PND45 (**Figure 48**).

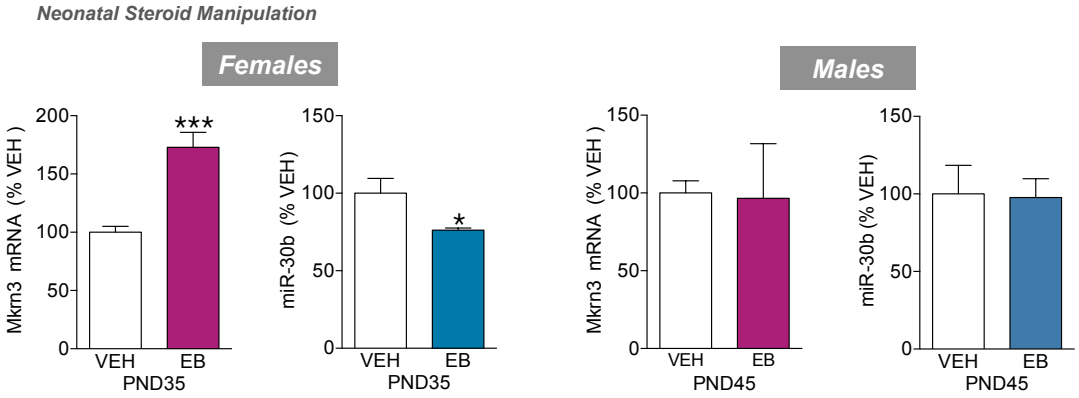


Figure 48: Expression profiles of Mkrn3 mRNA and miR-30b in the hypothalamus of male and female rats neonatally injected with estradiol benzoate (EB). Expression analyses were performed at the expected time of puberty, PND35 in female rats and PND45 in male rats. Animals injected with vehicle (olive oil, VEH) served as controls. Data are presented mean ± SEM (n = 5-8 animals/group). *, P ≤ 0.05, *** P ≤ 0.001 vs. VEH (Student t-test).

Model of photoperiodic manipulation: Constant darkness (CD) between PND10 and 15 disrupted puberty onset in both male and female rats, as evidenced by the delay in the age of BPS and VO, respectively (data not shown)[13]. Yet, no significant changes in *Mrkn3* mRNA or miR-30b levels were detected between control and CD animals during the juvenile-pubertal transition: PND15-35 in female rats and PND15-45 in male rats) (**Figure 49**).

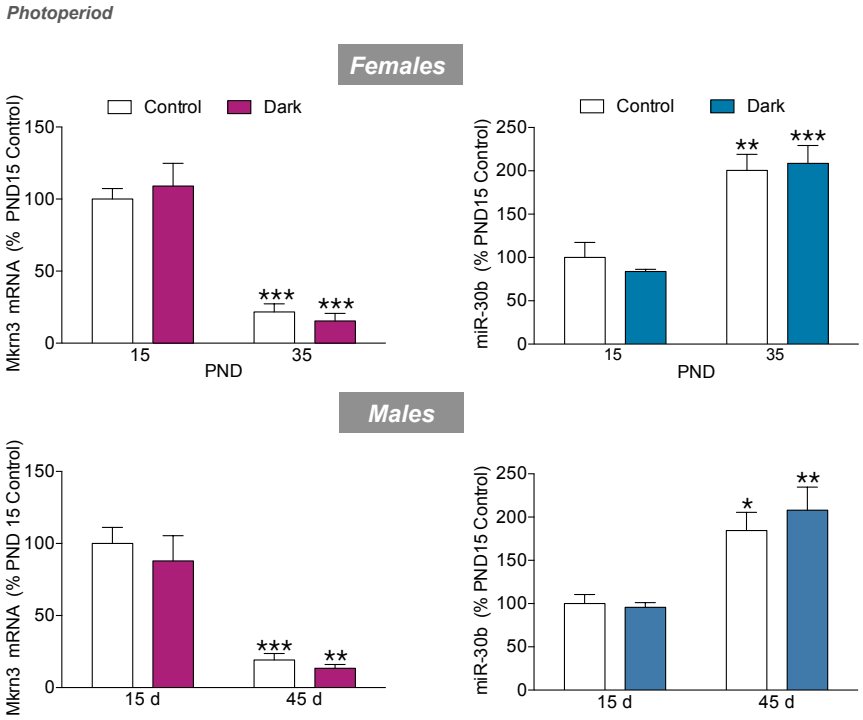


Figure 49: Expression profiles of Mkrn3 mRNA and miR-30b in the hypothalamus of male and female rats following photoperiod manipulation (CD, constant darkness from PND10–15). Expression analyses were performed during the juvenile-pubertal transition: PND15-35 in female rats and PND15-45 in male rats. Animals reared under standard photoperiodic conditions served as controls. Data are presented as mean ± SEM (n = 7-9 animals/group). * p ≤ 0.05, ** p≤0.01, ***p≤0.001 vs. PND-15 group for each photoperiodic regimen. Two-way ANOVA followed by post hoc Tukey test.

Model of early postnatal underfeeding: A protocol of undernutrition during lactation, generated by breeding pups in large litters (LL; 20 pups/litter), delayed the age of BPS and VO in male and female rats, respectively (data not shown), as reported elsewhere[13]. This pubertal phenotype was associated with alterations in the hypothalamic expression of Mkrn3 mRNA during postnatal development in early underfed female and male rats. Thus, enhanced expression of Mkrn3 was detected in LL female rats at PND5 and LL male rats at PND15 as compared with their respective controls (NL; normal litter), while no changes between NL and LL groups were observed at pubertal ages (PND 35 in females and PND45 in males). In contrast, hypothalamic expression of miR-30b was not significantly altered during pubertal development between NL and LL groups. Yet, a trend for a decrease in the hypothalamic levels of miR-30b was observed in LL female rats at PND5, as compared with their respective NL controls. In male rats, no significant alterations in miR-30b content were found between LL and NL groups during pubertal development (**Figure 50**).

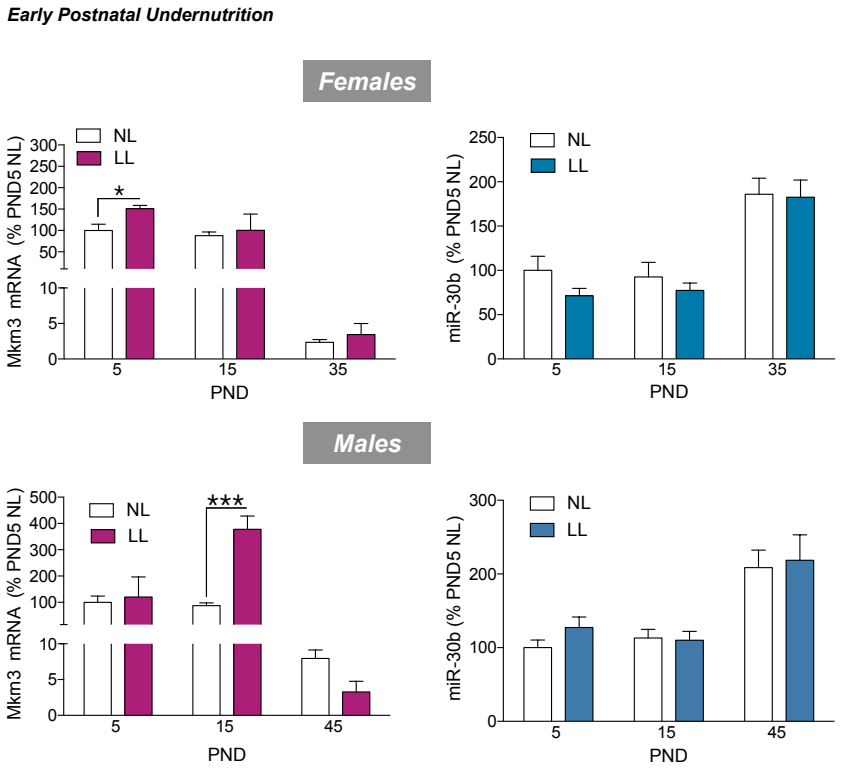


Figure 50: Expression profiles of Mkrn3 mRNA and miR-30b in the hypothalamus of male and female rats following early postnatal underfeeding. Expression analyses were performed at infantile (PND5), juvenile (PND15) and pubertal (PND35 in females and PND45 in males) ages. Data are presented as mean ± SEM (n = 6-8). **p≤0.01 vs. corresponding NL group at PND5, PND15 and PND35 (females) or PND45 (males) (Two-way ANOVA followed by Sidak's test).

HYPOTHALAMIC EXPRESSION OF MKRN3 PROTEIN DURING NORMAL POSTNATAL DEVELOPMENT AND IN CONDITIONS OF PERTURBED PUBERTY INDUCED BY NEONATAL ESTROGENIZATION

We also evaluated whether hypothalamic levels of Mkrn3 protein were altered in those animal models with most significant changes in Mkrn3/miR-30b ratios, namely (i) female pubertal development and (ii) neonatal steroid manipulation in female rats.

Consistent with Mkrn3 mRNA data, hypothalamic expression of Mkrn3 protein drastically declined during the juvenile-pubertal transition in female rats and significantly increased at the expected time of puberty in female rats neonatally injected with EB (**Figure 51**). These results indicate that alterations of hypothalamic Mkrn3 content also take place at posttranscriptional level in those animal models.

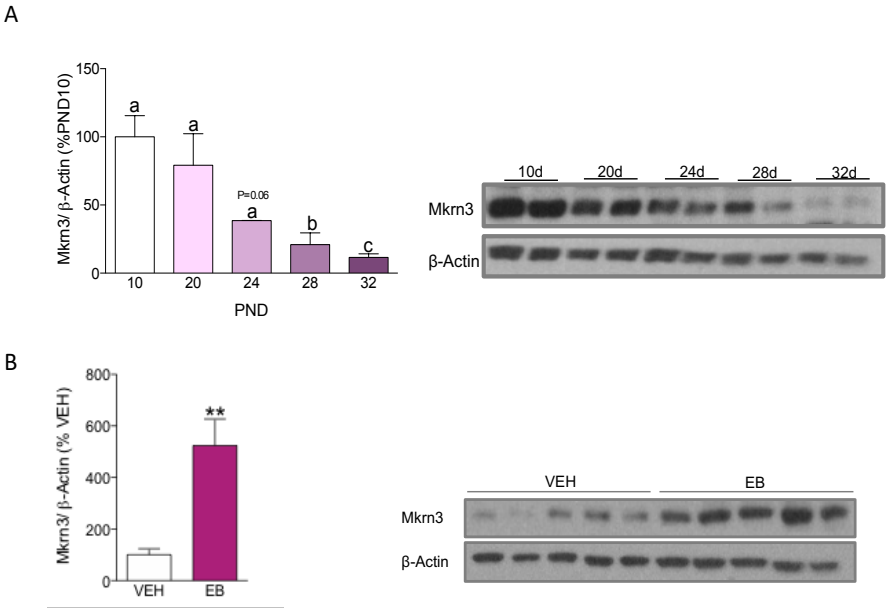


Figure 51: Densitometric quantifications (left panels) and representative western blot autoradiographic images (right panels) of hypothalamic Mkrn3 protein from female rats along postnatal development (A) and pubertal female rats (PND35) neonatally injected with estradiol benzoate (B) are shown. Animals injected with olive oil (VEH) served as controls in the model of neonatal estrogenization. Loading control (β-Actin) is also presented. Data are presented as mean ± SEM (n = 6-8 animals/group for densitometric quantifications in both experimental settings, postnatal development and neonatal estrogenization). For expression analyses along postnatal development, different superscript letters above bars indicate statistical differences (One-way ANOVA followed by post hoc Tukey test). For expression analyses in the neonatal estrogenization model, **P≤0.01 vs. VEH (Student t-test).

EVIDENCE FOR miR-30B MEDIATED REPRESSION OF MKRN3 FROM IN VITRO REPORTER ANALYSES

In order (i) to confirm in silico predictions that miR-30b targets the 3'UTR of Mkrn3 mRNA, and (ii) to document the potential inhibitory role of miR-30b on Mkrn3 expression suggested by the inverse Mkrn3/miR-30b ratios detected in specific animal models of normal and altered puberty, we

performed Luc-Pair miR luciferase assays by co-transfecting a reporter vector harboring the 3′-UTR of mouse Mkrn3 with a precursor expression plasmid for mouse miR-30b (3'-UTR + pre-miR-30b) in HEK 293 cells. As shown in **Figure 52**, co-transfection of pre-miR-30b with the reporter plasmid containing the 3'-UTR of Mkrn3 induced a dramatic reduction in the luciferase signal (>65%) compared to its control groups, which included the following plasmid combinations: 3'-UTR Mkrn3 + pcDNA (empty vector), 3'-UTR Mkrn3 + scrambled pre-miRNA, and scrambled 3'-UTR + pre-miR-30b. These data indicate that miR-30b targets the 3'-UTR of Mkrn3 and drives a repressive signal to Mkrn3 expression in vitro.

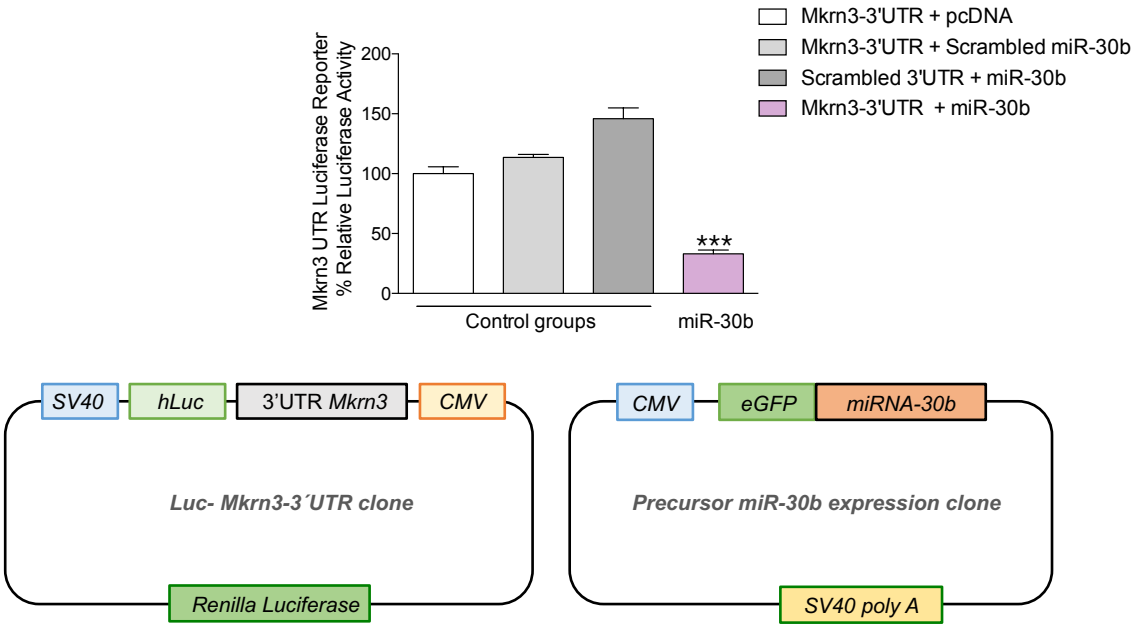


Figure 52: Luciferase activity of mouse Mkrn3 3′-UTR reporter construct after pre-miR-30b over-expression (3'-UTR Mkrn3 + pre-miR-30b) in HEK 293 cells (lower panel). The following combination plasmids were used as controls: 3'-UTR Mkrn3 + pcDNA (empty vector), 3'-UTR Mkrn3 + scrambled pre-miRNA, and scrambled 3'-UTR + pre-miR-30b. In addition, schematic representation of 3′-UTR target clone is shown (upper panel). Data are presented as mean ± SEM (n = 3 replicates/group). ***P≤0.001 vs each control group (One-way ANOVA followed by post hoc Tukey test).

TIMED CENTRAL ADMINISTRATION OF TSB-MIR-30b DELAYS PUBERTY ONSET IN FEMALE RATS

Based on the above data, we evaluated whether miR-30b would operate as a repressor of the hypothalamic expression of Mkrn3 in vivo, and thereby contribute to the central control of puberty. To test this hypothesis, we used antisense-modified oligonucleotides (namely, Target Site Blocker, TSB-miR-30b) that selectively prevent miR-30b binding to its seed regions at the 3′-UTR of Mkrn3. The impact of the central administration of a combination of three TSB-miR-30b, with capacity to prevent the binding of miR-30b to its seed regions at the 3'-UTR of Mkrn3, on the timing of puberty was evaluated in immature female rats (**Figure 53**). Two windows of treatment were selected: (i)

prepubertal, in which the TSB-miR-30 mix was icv injected at PND 24, 28 and 32 and (ii) *juvenile*, in which the TSB-miR-30 mix was icv injected at PND15, 22, 25 and 28.

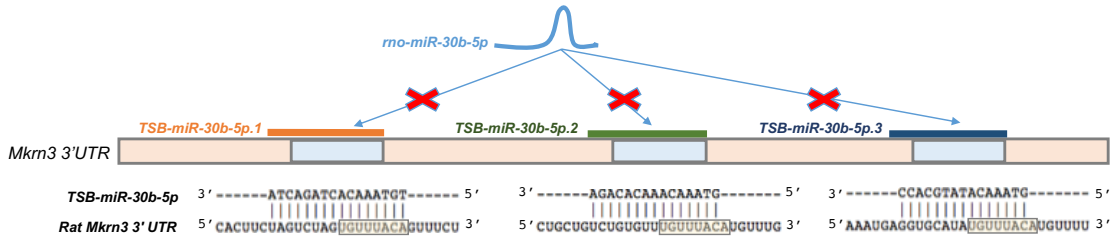


Figure 53: Schematic diagram showing the strategy used to selectively prevent the binding of miR-30b to its seed regions (underlined in light orange) at the 3'-UTR of Mkrn3.

Prepubertal TSB-miR-30 treatment did not modify relevant pubertal and/or metabolic parameters, including ages at vaginal opening or first estrus, relative ovarian and uterus weight, as well as body weight gain and food intake (**Figure 54**).

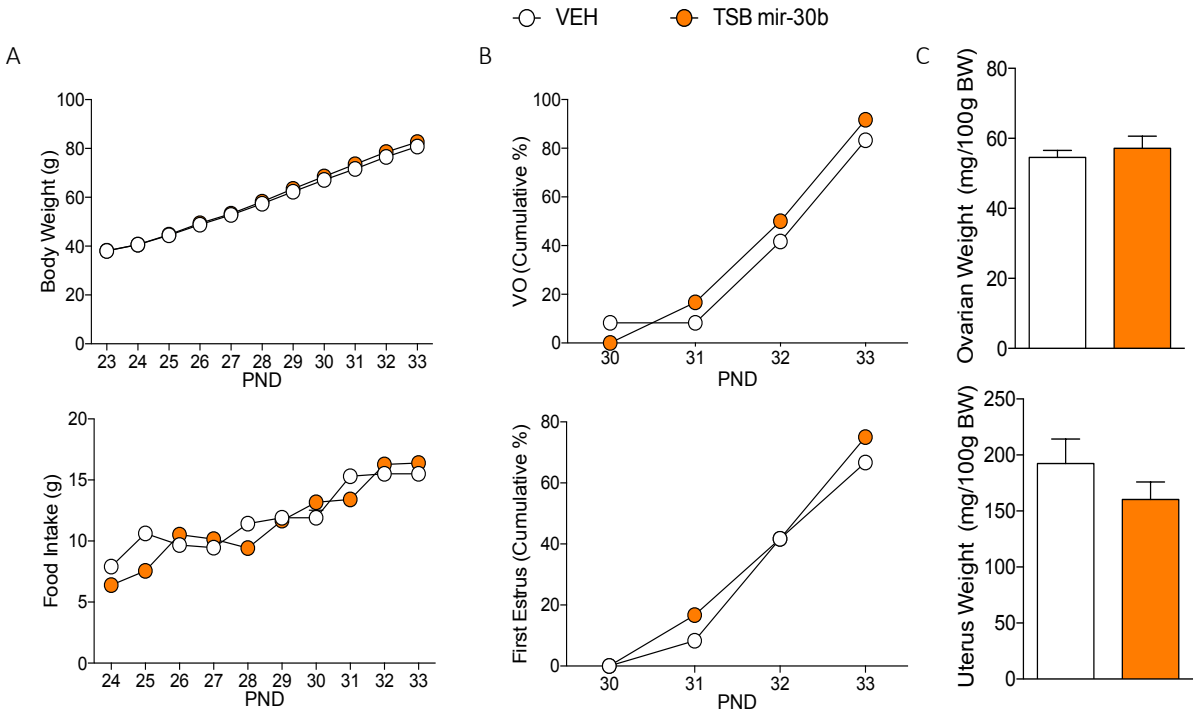


Figure 54: Impact of prepubertal icv administration of TSB-miR-30 on the timing of puberty onset in female rats. The effects of prepubertal icv treatment of TSB-miR-30 on body weight (BW) and food intake (A), as well as relevant reproductive parameters, including the cumulative percentage of vaginal opening (VO) and first estrus (B), and relative ovarian weight (OW) and uterus weight (UW) (C) are represented. Females icv injected with vehicle (VEH) served as controls (n = 10-12 animals/group).

In good agreement, no changes in the hypothalamic levels of Mkrn3 protein were observed in TSB-miR30 animals at the time of puberty onset (PND33) (**Figure 55**).

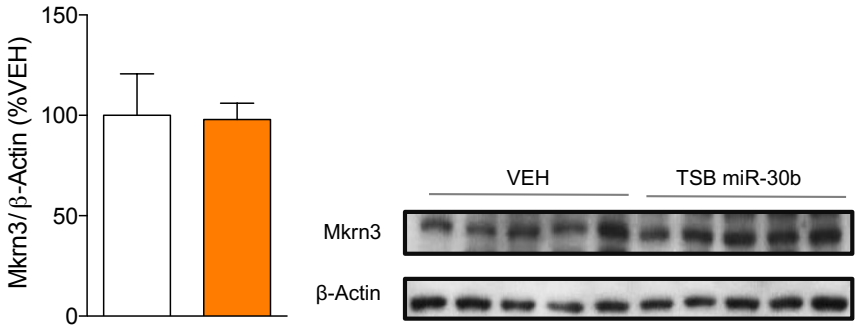


Figure 55: Densitometric quantifications and representative western blot autoradiographic images of Mkrn3 protein from hypothalamic samples of pubertal female rats subjected to prepubertal icv administration of TSB-miR-30b are shown Loading control (β-Actin) is also presented. Females icv injected with vehicle (VEH) served as controls (n = 5/group).

In clear contrast, juvenile TSB-miR-30b treatment resulted in delayed onset of puberty, as evidenced by the decreased percentage of animals (~50%) that displayed VO and first estrus when compared with its control group (**Figure 56**). Alike, a delay in follicular maturation and ovulatory scores was observed in the ovary (**Figure 57**).

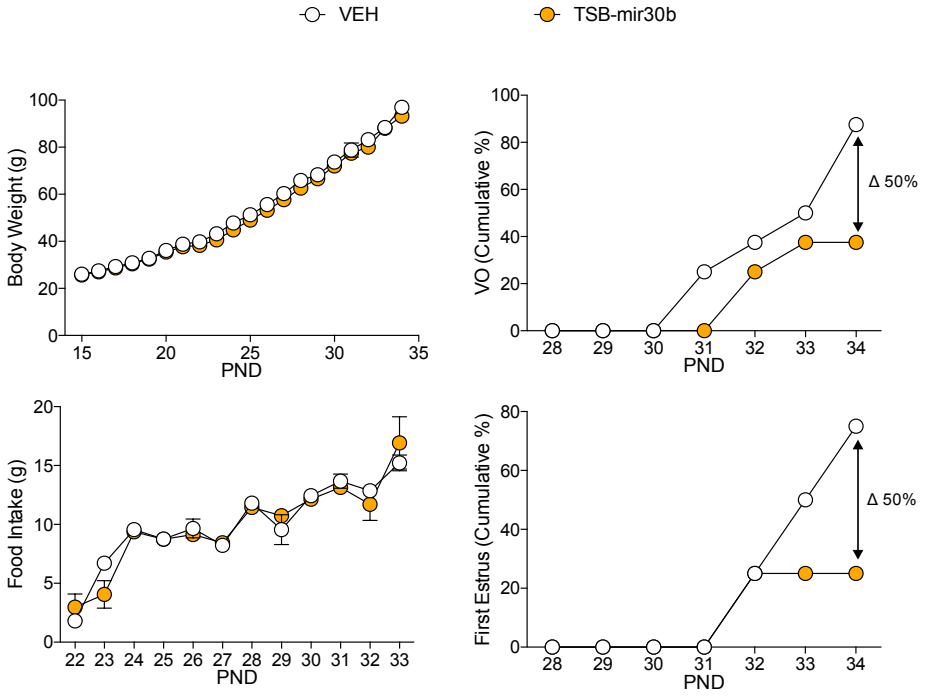


Figure 56: Impact of juvenile icv administration of TSB-miR-30 on the timing of puberty onset in female rats. The effects of juvenile icv treatment of TSB-miR-30 on body weight (BW) and food intake (left panels), as well as relevant reproductive parameters, such as the cumulative percentage of vaginal opening (VO) and first estrus (right panels) are represented. Females icv injected with vehicle (VEH) served as controls (n = 10-12 animals/group).

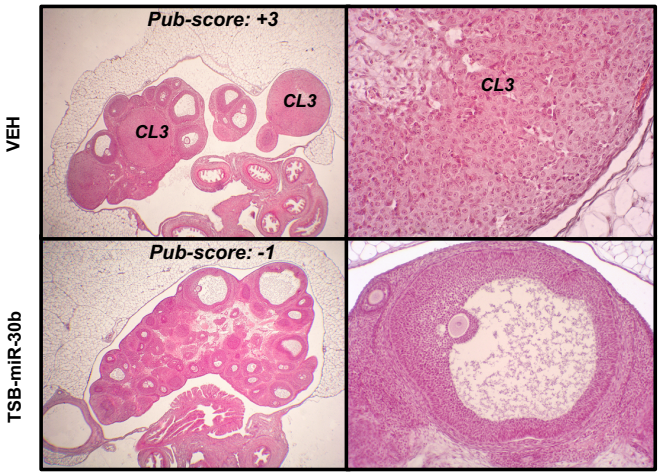


Figure 57: Representative images of ovarian maturation from pubertal female rats subjected to juvenile icv administration of TSB-miR-30b and its corresponding histological score of follicular development/ovulation (Pub-score; see *"Material and Methods"* section for further information) are shown.

Furthermore, this pubertal phenotype was linked to increased hypothalamic levels of Mkrn3 protein (**Figure 59**) and a trend for higher serum LH concentrations at PND34 (**Figure 58**), when most of the control animals showed VO and first estrus. Yet, no significant changes either in body weight or food intake were associated with such pubertal delay (**Figure 56**).

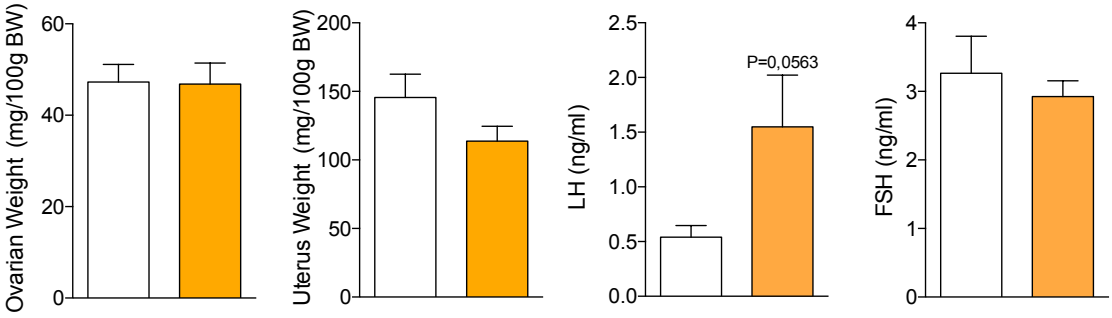


Figure 58: Impact of juvenile icv administration of TSB-miR-30 on relevant reproductive parameters, including relative ovarian (OW) and uterus weight (UW), as well as LH and FSH levels, in pubertal female rats (PND34) are represented. Females icv injected with vehicle (VEH) served as controls (n = 10-12 animals/group).

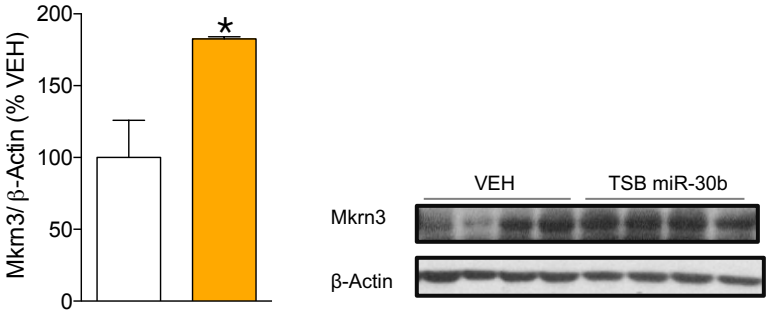


Figure 59: Densitometric quantifications and representative western blot autoradiographic images of hypothalamic Mkrn3 protein from pubertal female rats subjected to juvenile icv administration of TSB-miR-30 are shown. Loading control (β-Actin) is also shown. Females icv injected with vehicle (VEH) served as controls (n = 4/group). *P≤0.05 vs VEH group (Student t-test).

DISCUSSION

DISCUSSION

Puberty is a crucial developmental period that culminates with the attainment of reproductive capacity and sexual and somatic maturation[1]. This maturational process is set in motion at early stages of development and critically depends on the dynamic interplay between genetic and environmental factors. In fact, the disruption of this delicate interplay results in altered development of the neural networks responsible for pubertal control, and hence leads to perturbed timing of puberty[81]; a phenomenon that has been associated with increased risk for different types of cancer and severe metabolic, cardiovascular, neurological and reproductive disorders[277-280,281].

Nowadays, it is well known that (i) the initiation of puberty requires a sustained increase in the neurosecretory activity of GnRH neurons[41,48]; (ii) the pubertal rise in GnRH secretion is determined by coordinated changes in transsynaptic and glial inputs to the GnRH neuronal network[79]; and (iii) the development of the hypothalamic GnRH network depends on environmental factors, which are not only critical for puberty to occur, but also for its timing[112]. However, our current knowledge about the targets and mechanisms responsible for the central control of puberty and their contribution to alterations in the timing of puberty remains incomplete. In this context, the identification and characterization of novel modulatory pathways are crucial steps, since they may provide a better understanding of the neurobiological basis of puberty and would pave the way for the potential treatment of pubertal disorders.

To our knowledge, the involvement of brain lipid signaling in the physiological and patho-physiological control of puberty has never been addressed. Likewise, while very recent evidence suggests the potential role of the ubiquitin-protein ligase Mkrn3 in the hypothalamic regulation of puberty, the molecular mechanisms involved in its regulation and their biological actions in normal and altered puberty have not been investigated so far.

Based on the above, this Doctoral Thesis has explored (i) the role of a family of sphingolipids, named ceramides, in the hypothalamic control of puberty and its potential contribution to obesity-induced precocious puberty; and (ii) the involvement of the hypothalamic miR-30b/Mkrn3 pathway to the timing of puberty and its potential alterations due to adverse environmental conditions.

With the aim to facilitate the discussion of our findings, this section has been divided into two major subsections, corresponding to the main objectives mentioned above.

CENTRAL CERAMIDE SIGNALING & PUBERTY

Recent studies have suggested a link between the rising prevalence of childhood obesity and a decline in the age of puberty, especially in girls[106,282,283]. However, the potential targets and underlying mechanisms for this association are still unknown. During the last two decades, much has

been gleaned about the neuroendocrine mechanisms responsible for the tight coupling between energy homeostasis and puberty onset. These mechanisms seemingly involve a plethora of metabolic hormones and neuropeptides, which impinge and integrate at the hypothalamic centers governing reproduction[3,4]. Among those regulatory signals, the metabolic hormone leptin, as a permissive/ stimulatory factor of puberty[6,129,136], and the puberty-activating neuropeptide, kisspeptin, as a central conduit for transmitting the pubertal actions of different metabolic hormones to the reproductive brain, have proven to be especially relevant[5-7].

Interestingly, data from preclinical animal models strongly suggested that perturbations of hypothalamic signaling pathways may contribute to the alterations in the timing of puberty induced by early overnutrition. In particular, increased hypothalamic expression of kisspeptin has been linked to advanced puberty in both female rats and mice subjected to different protocols of early overnutrition[161,164,284,285]. In contrast, other studies conducted in similar models of nutritional manipulation failed to detect this association[163,164]. These findings illustrate the complexity of the potential mechanisms that may underlie obesity-induced precocious puberty, which may involve not only the neuro-hormonal actions of kisspeptin but also the contribution of alternative targets and/or regulatory pathways.

In addition to neuropeptides, different molecular and cellular mediators are emerging as relevant elements in the central control of reproduction and/or energy balance. Among them, the sphingolipids ceramides seem to have a prominent role in metabolic homeostasis[8,9]. Ceramides are involved in a wide range of biological processes, including cell growth, differentiation, and apoptosis[286]. Of note, recent studies have shown their relevant roles as putative mediators of different metabolic disorders[8,9] and transmitters for the central actions of leptin and ghrelin[11,12], two key hormones for the regulation of metabolism and puberty onset. In particular, increased hypothalamic levels of ceramides have been shown to block the anorectic actions of leptin, while leptin decreases ceramide synthesis[11]. In turn, ghrelin upregulates ceramide synthesis, which is mandatory for its orexigenic actions[12]. Whether central ceramide signaling exerts a relevant role in the central control of puberty and underlies obesity-induced precocious puberty remains totally unexplored. The research findings presented in this Doctoral Thesis demonstrate a novel regulatory role of central ceramide signaling in the control of puberty onset and provide solid evidence for its contribution to the pathophysiology of precocious puberty linked to early overfeeding.

In this context, our data are the first to document that the hypothalamic levels of ceramides are significantly increased in early overfed female rats that display advanced puberty. In line with these results, a higher concentration of hypothalamic ceramides has also been reported in adulthood in other animal models of overnutrition and/or obesity, including mice subjected to HFD[287] and obese Zucker rats[10,288]. However, its impact on the timing of puberty has not been addressed so far. To our knowledge, these findings are the first to suggest that an increase in the hypothalamic content of

ceramides may contribute to the pathophysiology of precocious puberty linked to early overfeeding.

In addition to the hypothalamic rise in ceramides levels, early overnutrition and/or obesity are often associated with severe deregulation of endocrine axes and neurohormonal pathways, which could affect "per se" the timing of puberty. To avoid the potential influence of those confounding factors on pubertal timing and in order to address the specific impact of central ceramide signaling on the control of puberty onset, we centrally (icv) manipulated the hypothalamic levels of ceramides in lean female rats during the prepubertal period. Our results show that chronic stimulation of central ceramide synthesis with CER C6, a cell-penetrating ceramide precursor, results in advanced vaginal opening and first ovulation, while its persistent blockade with MYR, an inhibitor of the *de novo* ceramides synthesis, causes the opposite effect; that is, delayed puberty. These data not only support the contribution of enhanced ceramide signaling to the phenotype of precocious puberty previously suggested in conditions of overnutrition but also suggest the potential regulatory role of central ceramide signaling on the physiological timing of puberty in female rats. Of note, central ceramides have been proposed as potential mediators in the control of energy balance in adult rats[11,12,289]. However, our data show that its pharmacological manipulation during the pubertal development does not affect relevant metabolic parameters linked to energy homeostasis, such as food intake and body weight. This phenomenon stresses the specific regulatory role of central ceramide signaling in the central control of puberty, which does not seem to be related to its potential actions in the control of energy balance.

The mechanism whereby central ceramides might influence the timing of puberty seems to involve the interaction with kisspeptin, and, possibly to a lesser extent, also leptin. This is based on the observed impact of icv administration of kisspeptin and leptin on the timing of puberty onset after central blockade of ceramide signaling in prepubertal female rats subjected to chronic under-nutrition; a murine model of delayed puberty that is associated with a reduced hypothalamic content of kisspeptin and lower peripheral levels of leptin[3,112]. In particular, we found that central blockade of ceramide signaling largely prevented the stimulatory effects of kisspeptin on puberty onset (44% reduction of VO) and LH secretion. However, such blockade in ceramide signaling only modestly suppressed the permissive actions of leptin on the onset of puberty (11% of VO suppression), and to a large extent, the first estrus. These findings suggest that central ceramide signaling might mediate part of the stimulatory/permissive effects of kisspeptin and, to a lesser degree, leptin on puberty onset. Of note, central administration of leptin has been shown to reduce the hypothalamic levels of ceramides in adult male rats; a phenomenon that seems to mediate the anorexigenic actions of leptin[11]. Whether such leptin treatment modulates the timing of puberty through the suppression of hypothalamic ceramides levels in our experimental model is an intriguing question that has not been addressed. However, it seems unlikely that the major modulatory actions of leptin on puberty onset are ceramide-dependent due to the modest impact of the central blockade of ceramide signaling on

its pubertal actions.

The above results support that hypothalamic ceramides might contribute to the regulation of puberty through their interaction with with kisspeptin signaling, which is a master regulator of gonadotropin secretion. However, our data also suggest that the actions of ceramides on the central control of female puberty might be more complex and to a large extent gonadotropin-independent. This is illustrated by our in vivo and in vitro experiments of pharmacological manipulation of central ceramide signaling. Interestingly, the alterations in the timing of puberty induced by chronic icv treatment with CER C6 or MYR were not associated with consistent changes in gonadotropin secretion. In the same line, the challenge of hypothalamic and pituitary explants from immature female rats with either CER C6 or MYR failed to alter GnRH or gonadotropin secretion, respectively. These data suggest that central ceramide signaling might contribute to the modulation of puberty through alternative regulatory pathways that might complement the neuroendocrine mechanisms classically involved in the control of puberty onset. Indeed, the lack of impact of the central blockade of ceramide signaling on (i) the hypothalamic expression of Kiss1, (ii) kisspeptin-induced GnRH/gonadotropin secretion, and (iii) GnRH-induced gonadotropin release in immature female rats, supports such intriguing possibility.

In addition to the classical neurohormonal control of the gonadotropic axis, increasing evidence supports that the brain exhibits a neural control over ovarian function[46,65]. In particular, the hypothalamic PVN nucleus has been proposed as the main hypothalamic region for transmitting sympathetic neural information to the ovary[290]. This information is conveyed from the PVN to preganglionic sympathetic neurons first, and then to celiac postganglionic cells, which are responsible for innervating the ovary[290]. Considering our data and the limited evidence supporting the influence of central ceramide signaling on the neuroendocrine control of puberty, we explored whether hypothalamic ceramides might influence the timing of puberty onset through modulation of an alternative PVN-ovarian sympathetic pathway that might contribute to obesity-induced precocious puberty.

In support of a putative role of deregulated ovarian sympathetic modulation in the pathogenesis of obesity-induced precocious puberty, our results document a significant increase in the content of NE and MHPG, two relevant markers of sympathetic activity[291], in the celiac ganglion and the ovary of early overfed animals. Furthermore, we also found that the ovarian expression of Ngf, a surrogate marker of the sympathetic tone of the ovary[74], was enhanced in those animals. Because a significant elevation in ovarian NE content has been documented in lean female rats when puberty approaches[292], these findings suggest a premature activation of ovarian sympathetic activity in those animals, which might be associated with an advance in the age of puberty onset.

Our experiments of icv manipulation of central ceramide signaling in early overfed female rats during pubertal maturation are in line with this hypothesis. Thus, our data show that the central

blockade of ceramide signaling with MYR partially normalized the timing of puberty onset in early overfed female rats and that such phenomenon is associated with a partial normalization of the ovarian sympathetic tone in those animals. Of note, while the content of NE in the celiac ganglia and the ovary was completely normalized in early overfed animals treated with MYR, the levels of MHPG were not restored. The latter suggests that the normal release of ovarian NE was not fully recovered by MYR treatment in early overfed animals, which is consistent with the partial, but not total, normalization of the timing of puberty observed in those animals. Perhaps a more extended period of treatment with MYR might completely normalize both the onset of puberty and the ovarian sympathetic tone in those animals. Moreover, the fact that ovarian Ngf signaling was significantly downregulated by MYR in early overfed animals is also suggestive of the role of central ceramide signaling in the control of ovarian sympathetic tone, and thereby pubertal timing. In fact, a decreased Ngf expression at early stages of development has been associated with low NE activity and poor ovarian nerve development[74,293] . All in all, these findings suggest that a perturbed central ceramide signaling might contribute to the pathophysiology of precocious puberty linked to early overfeeding through alterations in the ovarian sympathetic activity.

Remarkably, our data suggest also that the hypothalamic modulation of ovarian sympathetic activity in early overfed female rats with precocious puberty might result from alterations in the synthesis of ceramides at the PVN. Interestingly, a discernible expression of SPTLC1, a key enzyme for the *de novo* ceramide synthesis, was detected in the PVN of lean female rats when approaching puberty. Furthermore, such SPTLC1 expression was significantly increased in the PVN of early overfed rats with precocious puberty. These results are in good agreement with the higher hypothalamic levels of ceramides detected in those animals, and stress the potential relevance of changes in ceramide synthesis at the PVN in the alterations of ovarian sympathetic activity linked to obesity and precocious puberty. Yet, functional studies are required to fully validate such hypothesis. Additionally, it would be also important to evaluate the potential interaction between kisspeptin and central ceramides at the PVN in the modulation of the ovarian sympathetic activity, as putative modulator of the onset of puberty. Of note, such potential interplay would be compatible with our findings of prevention of kisspeptin effects in our model of pharmacological blockade of central ceramide synthesis. Interestingly, our data show that the number of PVN kisspeptin-ir fibers is drastically reduced in obese female rats with advanced puberty, which is in line with the declining trend in kisspeptin-ir detected in pubertal female mice subjected to early overnutrition[162]. Of note, such a decrease in PVN kisspeptin-ir may not necessarily translate into an equivalent reduction in neuropeptide release. On the contrary, a reduction in kisspeptin content may stem from an increase release of the peptide. Indeed, a significant increase in the hypothalamic kisspeptin tone has been detected in different animal models of early overnutrition linked to precocious puberty[161,164,284,285]. Whether those alterations in PVN kisspeptin-ir are functionally related with changes in PVN ceramide

signaling and may influence the ovarian sympathetic activity (and thereby pubertal timing) is an intriguing question that requires further investigation. Yet, it is important to stress that changes in kisspeptin-ir according to the phases of the estrous cycle have been observed in the PVN of adult female mice[265]; a phenomenon that suggests the potential physiological relevance of those alterations.

In summary, our studies document a novel regulatory role of central ceramide signaling in the control of pubertal timing and its alteration due to early-onset obesity. Furthermore, our data support a potential interplay of central ceramides with kisspeptin in the modulation of puberty and disclose an alternative pathway, linking the synthesis of ceramides at the hypothalamic PVN nucleus and the sympathetic innervation of the ovary, as key for obesity-induced precocious puberty.

miR-30b/Mkrn3 SYSTEM & PUBERTY

A large body of evidence suggests that MKRN3, a maternally imprinted gene encoding the makorin RING-finger protein 3, operates as a potential repressor for the control of puberty, since (i) deleterious mutations of Mkrn3 have been associated with central precocious puberty in boys and girls[16-22], (ii) serum levels of Mkrn3 decrease before puberty onset in both sexes[23-25], and (iii) the hypothalamic expression of Mkrn3 is significantly reduced before the onset of puberty in mice[16,26]. However, the molecular mechanisms for the biological actions and regulation of Mkrn3 in normal and altered puberty remain virtually unknown.

Interestingly, the analysis of the 3'-UTR region of MKRN3 transcript, a key element for miRNA-mediated post-transcriptional regulation of gene expression, reveals a 90% of identity between mice and humans[255]; a particular genetic feature that not only suggests the functional significance of this region for the biological actions of MKRN3, but also the potential role of miRNAs with ability to bind to specific sequences (seed regions) located at this 3'-UTR in the regulation of MRKN3 activity[255]. However, this intriguing possibility has not been explored so far. The studies performed in this Doctoral Thesis have addressed this question and provide solid evidence for a relevant regulatory role of the microRNA, miR-30b, in the control of the hypothalamic expression of Mkrn3 and its functional involvement in the central modulation of puberty.

Our in silico analyses of the 3′-UTR of MKRN3 gene in humans, mice and rats identified a significant number of predictive and conserved binding sites for the members of the miRNA family, miR-30, in this gene region, thus suggesting its potential role in the regulation of MKRN3 expression. Among them, we focused our attention on the microRNA, miR-30b, based on three major reasons: (i) its documented expression in rat hypothalamus[274], (ii) its demonstrated regulation by sex steroids[276,294] and, (iii) the high number of predictive and conserved binding sites, three, detected for this miRNA in the 3'UTR of MKRN3 gene.

Consistent with previous studies in mice[16,26], the hypothalamic expression of Mkrn3 mRNA and protein decreases during postnatal maturation before puberty in rats, supporting the concept that a reduced hypothalamic tone of Mkrn3 is permissive for puberty to proceed. Interestingly, we found that the hypothalamic content of miR-30b was inversely correlated with the expression pattern of Mkrn3; that is, low levels during the infantile period and increasing levels thereafter in both sexes. Considering that miRNAs have the capacity to bind to partially complementary sequences in the 3'-UTR of target genes and induce its translation blockade and silencing[187], this inverse correlation suggests that miR-30b might be a potential repressor of the hypothalamic expression of Mkrn3 during pubertal maturation. Furthermore, this observation supports the idea that miR-30b and Mkrn3 might represent a potential regulatory pathway in the central control of puberty.

To provide further evidence for the putative regulatory role of miR-30b/Mkrn3 pathway in the development of the hypothalamic systems leading to the initiation of puberty, we conducted a series of expression analyses in both male and female rats using three different models of perturbed puberty: (i) neonatal estrogenization, (ii) early alteration of photoperiodic conditions and (ii) early postnatal underfeeding. Consistent with previous studies, the above manipulations disrupted the onset of puberty in both sexes[13,270]. Interestingly, neonatally estrogenized female rats showed enhanced hypothalamic levels of Mkrn3 mRNA and protein at the expected time of puberty, while the hypothalamic expression of miR-30b was downregulated in those animals. A similar alteration of the hypothalamic miR-30b/Mkrn3 ratio was also observed in early underfed female rats at the early infantile period (PND5), but not at later ages, including the expected time of puberty. In contrast to the above manipulations, female rats subjected to constant darkness between PND10 and PND15 did not show significant changes in the hypothalamic Mkrn3 mRNA or miR-30b expression when compared with their respective controls. In addition to support the potential regulatory role of miR-30b on the hypothalamic Mkrn3 expression, these results suggest that an adequate hypothalamic miR-30b/Mkrn3 ratio, especially at early stages of development, might be relevant for the proper timing of puberty in female rats[255].

Furthermore, the variable impact of the above manipulations on the hypothalamic expression of miR-30b and Mkrn3 might indicate that the miR-30b/Mkrn3 hypothalamic pathway is more sensitive to certain environmental conditions, including altered sex steroid milieu or nutritional stress, than others, such as perturbed photoperiodic conditions. Indeed, compelling, but still fragmentary, evidence suggests the potential influence of sex steroid and nutritional environment on MKRN3 peripheral levels and miR-30b expression. Thus, a recent study shows that MKRN3 circulating levels decreases in girls with central precocious puberty (CPP) and that such decline is associated with an increase in the peripheral levels of estrogen and a higher Body Mass Index (BMI)[295], considered as a marker of nutritional status. In the same vein, miR-30b has been proposed as an estrogen- and nutritional-sensitive miRNA[274-276,294]. Thus, miR-30b expression is enhanced in breast cancer cells

after estradiol treatment[294], fluctuates in human endometrium through different physiological phases[275] and increases in the hypothalamus of male rats subjected to HFD during 3 months after weaning[274]. All in all, the above data are in good agreement with our current results and suggest that the miR-30b/Mkrn3 pathway may be particularly sensitive to sex steroids and nutritional environment. Yet, it is worth to note that our experiments, unlike previous studies, addressed the impact of the early manipulation of those environmental conditions on the hypothalamic expression of miR-30b and Mkrn3 during pubertal maturation. Therefore, the hypothalamic disruption of miR-30b/Mkrn3 pathway detected in our experimental settings might be the result of an early mal-programming of the brain circuits that control the onset of puberty, rather than acute responses to a transient exposure to high levels of estrogen or a reduction in food intake. In any case, the specific contribution of the hypothalamic disruption of miR-30b/Mkrn3 pathway to the altered puberty observed in our experimental animal models requires further investigation.

Strikingly, unlike females, no alterations in the hypothalamic miR-30b/Mkrn3 ratios were detected in the hypothalamus of male rats subjected to neonatal estrogenization and early postnatal undernutrition, as compared with their respective controls. This phenomenon suggests that, even though early disruption of brain sexual differentiation or nutritional stress causes pubertal alterations in both male and female rats, the involvement of miR-30b/Mkrn3 pathway in such alterations is likely different between both sexes. The physiological relevance for this sexual dimorphism remains to be established.

The importance of the hypothalamic miR-30b/Mkrn3 pathway in the physiological control of puberty was attested by our in vivo studies, using timed icv administration of tailored TSB to selectively prevent binding of miR-30 to its conserved seed regions at the 3'-UTR of Mkrn3 at central levels. Thus, blocking experiments, involving TSB injections during the juvenile phase (between PND15 to 28) unambiguously documented that prevention of the repressive action of miR-30 on the 3'-UTR of Mkrn3 not only attenuates the decline of the hypothalamic content of Mkrn3 during the juvenile-pubertal transition, but causes also a significant delay in the onset of puberty and partially suppresses ovarian maturation. This pubertal phenotype was associated with an unexpected increase in LH levels; a phenomenon that may be explained as a compensatory response to the predicted low levels of estradiol in TSB-miR-30 animals, as suggested by the reduced relative uterine weights observed in those animals. Strikingly, this effect appeared to be developmentally-sensitive, as a similar protocol of target-site blocking applied during the pre-pubertal transition (between PND24 to 32) failed to cause significant changes in hypothalamic Mkrn3 content or pubertal timing. These findings suggest the participation of the hypothalamic miR-30/Mkrn3 pathway, during discrete maturational windows, in the programming of brain circuits later involved in the control of puberty onset; a possibility that is in line with results from our preclinical models of altered puberty due to early manipulations of the sex steroid or nutritional milieu, in which alterations in the miR-

30b/Mkrn3 ratios were detected. Of note, while our expression analyses focused on miR-30b, we cannot rule out the potential involvement of additional miRNAs in the hypothalamic control of Mkrn3 expression, as our TSB strategy prevents the binding of all miR-30 family members to the 3'-UTR, and our in silico analyses highlighted also other miRNAs with ability to bind to specific seed regions at the Mkrn3 gene.

The neuroendocrine mechanism(s) whereby miR-30b/Mkrn3 pathway may contribute to the control of puberty is still a relevant question that remains to be resolved. Recent evidence suggests that Mkrn3 may modulate the onset of puberty through a mechanism that involve the ubiquitination and, eventually, suppression of a relevant protein involved in neural differentiation, neural pentraxin-1 precursor (Nptx1)[26]. Yet, no evidence for the influence of Mkrn3/Nptx1 pathway on the gonadotropic axis has been provided. Although our studies did not address this intriguing question, the neuro-anatomical distribution of Mkrn3 protein, which is overrepresented at the hypothalamic arcuate nucleus, may suggest its potential interaction with relevant puberty-related neuropeptides that are highly expressed in such hypothalamic region, including the key components of KNDy neurons: Kisspeptin, Neurokinin B and Dynorphin. Whether miR-30b/Mkrn3 pathway regulates the onset of puberty through its interaction with those or other puberty-related neuropeptides is an intriguing possibility that requires further investigation. Regarding the transcriptional regulation of Mkrn3 expression, a very recent work has suggested that the transcription factor named downstream responsive element antagonist modulator (DREAM), predicted to bind to the 5'-UTR of Mkrn3 gene, may participate in the control of the transcriptional activity of Mkrn3[296]. Yet, while disruption of such regulatory site may be pathogenic for central precocious puberty, direct evidence for DREAM/Mkrn3 interaction or its capacity to regulate Mkrn3 expression is yet to be presented. Interestingly, very recent evidence has preliminarily documented an interplay between miR-30 and MKRN3 in the context of gastric cancer[297], while miR-30 has been shown to modulate ubiquitin E3 ligase activity in ovarian cells[298].

Our studies adds to those previous data, illustrating the capacity miR-30b to modulate, acting at highly conserved regulatory regions of its 3'-UTR, Mkrn3 expression, and proving the relevance of such action on the central control of puberty. Moreover, our data suggest that a correct functioning of miR-30b/Mkrn3 pathway at early stages of development is essential for the normal initiation of puberty and pave the way for a better understanding of the basis for altered puberty in conditions of metabolic stress and/or hormonal disruption.

GRAPHICAL SUMMARY

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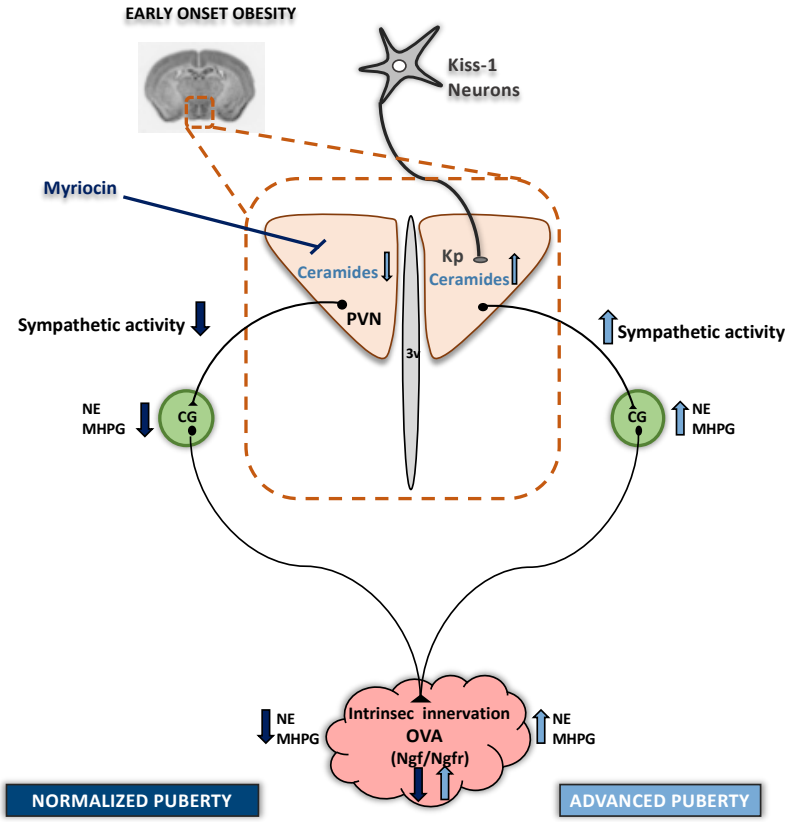
The major findings of the present PhD Thesis can be summarized as follows:

1. *Central ceramide signaling plays a novel regulatory role in the control of pubertal timing and its alteration due to early-onset obesity*. In detail, we found that: (i) hypothalamic levels of ceramides are increased in early overfed female rats with precocious puberty; (ii) chronic stimulation of central ceramide synthesis with CER C6, a cell-penetrating ceramide precursor, advances the onset of puberty in female rats, while its persistent blockade with MYR, an inhibitor of the *de novo* ceramide synthesis, causes delayed puberty; and (iii) central ceramide signaling mediates part of the stimulatory/ permissive effects of kisspeptin and, to a lesser degree, leptin on puberty onset. Furthermore, our data disclose an *alternative* pathway, linking kisspeptin projections and ceramide synthesis at the PVN with the sympathetic innervation of the ovary, as key for obesity-induced precocious puberty. Specifically, we found that: (i) immature female rats subjected to early overfeeding show a premature increase in the ovarian sympathetic activity; (ii) central blockade of ceramide signaling partially normalizes the timing of puberty in early overfed female rats through restoring their ovarian sympathetic tone; and (iii) immunoreactivity at the PVN for kisspeptin fibers and SPTLC-1, a key enzyme of the *de novo* synthesis of ceramides, is altered in early overfed rats with precocious puberty.



CONCLUSIONS

CONCLUSIONS

The major conclusions of this Doctoral Thesis are the following:

1. Ceramide signaling constitutes a novel pathway for the central control of pubertal timing, which mediates at least part of the regulatory actions of kisspeptins (and to a lesser extent leptin), likely via a GnRH-independent pathway, involving the PVN and ovarian sympathetic innervation.

2. Kisspeptin-ceramide pathway at the PVN plays a relevant pathophysiological role in the generation of pubertal precocity associated to early-onset obesity.

3. The miRNA, miR-30b, is a novel central regulator of the puberty-repressing factor, Mkrn3, acting at highly conserved regions at the 3'-UTR of the gene.

4. MiR-30b/Mkrn3 pathway seemingly plays a distinct role in the physiological control of the timing of puberty, and its perturbations in conditions of early nutritional or hormonal alterations.

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